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ENDOGENOUS LECTIN ACTIVITY IN THE DEVELOPING YOLK SAC MEMBRANE OF THE CHICKEN EMBRYO

BY

GERALDINE M. MBAMALU

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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OF MASTER OF SCIENCE

IN

ZOOLOGY

FIELD OF STUDY: CELL BIOLOGY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

SPRING, 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ENDOGENOUS LECTIN ACTIVITY IN THE DEVELOPING YOLK SAC MEMBRANE OF THE CHICKEN EMBRYO submitted by GERALDINE M. MBAMALU in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE IN ZOOLOGY, FIELD OF STUDY: CELL BIOLOGY.

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Date October 30 1985

ABSTRACT

The objective of this work was the detection, isolation and characterization of soluble, endogenous lectin activity from the early yolk sac of chicken embryos. Extracts from the area vitellina and area vasculosa, the precursors of the yolk sac which are in turn derived from the area opaca of the chick blastoderm, were studied. These tissues from embryos at stages 13, 14 and 15 contained lectin activity which was detected by an haemagglutination assay using trypsinized rabbit erythrocytes. Lectin mediated haemagglutination was inhibited by galactose-containing saccharides and the most potent saccharide inhibitors were thiodigalactoside, lactose and (2R)-glycerol-O-β-D-galactopyranoside. The lectin activity, which could be extracted from whole tissue in the presence of lactose, did not appear to require the presence of divalent cations, but did have a requirement for disulphide reducing agents for the maintenance of its activity.

Generally higher levels of lectin activity were present in the area vasculosa, as compared to the area vitellina; this was true in embryos up to seven days of incubation. The area vasculosa is the more differentiated of these two tissues and by seven days of development has normally enclosed the yolk.

Lectin activity was purified by affinity chromatography on a Sepharose 4B column to which the ligand, para-aminophenyl-β-D-lactoside was linked. Purified lectin preparations from the area vitellina and area vasculosa of stage 13-15 yolk sacs were examined by sodium dodecyl

sulphate-polyacrylamide gel electrophoresis. Under reducing conditions two main bands were observed; a more prominent band with a subunit molecular weight of about 14,200 ± 100 for the area vitellina and 13,700 ± 300 for the area vasculosa and a second band with a molecular weight of about 68,100 ± 700 and 68,700 ± 1200 for the area vitellina and area vasculosa, respectively. The high molecular weight protein sometimes appeared as a doublet with molecular weights of about 71,300 ± 1300 and 62,700 ± 1400 in the area vitellina lectin preparation. When crude preparations were examined by gel filtration chromatography under non-dissociating conditions and in the presence of lactose, the lectins appeared to be present as aggregates. In isoelectric focusing studies, the affinity-purified lectin(s) from the area vitellina and area vasculosa contained at least two distinct proteins. A major band was observed, with isoelectric points of 6.58 and 6.65 for the area vitellina and area vasculosa lectins, respectively. In addition, two to four minor bands were observed in the acidic pH range of 4.1 to 5.5 for the area vitellina lectin and 4.05 to 5.1 for the area vasculosa lectin.

The soluble, β-D-galactoside-binding lectins in the area vitellina and area vasculosa of stage 13-15 yolk sacs are considered to be very similar, if not identical proteins. The presence of lectin activity in the yolk sac tissues at progressive stages of development and the higher levels of lectin in the area vasculosa suggest a role for the lectin in the differentiation of this extraembryonic tissue.

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LIST OF ABBREVIATIONS

APL	para-aminophenyl-β-D-lactoside
A. Vasc.	Area vasculosa
A. Vit.	Area vitellina
Bis	N, N'-methylenebisacrylamide
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue R-250
Con A	Concanavalin A
EDTA	Ethylenedinitrilotetraacetic acid
GalNAc	N-acetyl-D-galactosamine
GlcNAc	N-acetyl-D-glucosamine
HU	Haemagglutinating units
LES	Lectin extraction solution
M	Molar
2ME	2-mercaptoethanol
MEPBS	Mercaptoethanol phosphate buffered saline
NaN_3	Sodium azide
PMSF	Phenylmethylsulphonyl fluoride
pps	Pulses per second
RCA	Ricinus communis agglutinin
RGG	(2R)-glycerol-O-β-D-galactopyranoside
SBA	Soybean agglutinin
SDS	Sodium dodecyl sulphate
SE	Standard error
TDG	Thiodigalactoside [β-D-gal(1-->1)β-D-thiogal]
TEMED	N, N, N', N'-tetramethylethylenediamine

Tris	Tris (hydroxymethyl)aminomethane
WGA	Wheat germ agglutinin

INTRODUCTION

1. THE YOLK SAC MEMBRANE

The yolk sac is one of three extraembryonic membranes necessary for the development of the chicken embryo. It is derived from the blastoderm and is continuous with the embryonic body. The yolk sac is the primary organ of nutrition, functioning in the uptake and digestion of nutrients from the yolk and their transport to the embryo. This structure has distinctive morphological and physiological characteristics consistent with its role in development (Romanoff, 1960).

1.1 Gastrulation in the Chicken Embryo

Gastrulation in the chicken embryo involves several morphogenetic processes directed by the genome of the developing embryo. During gastrulation, extensive cell rearrangments occur with cell groups present in discrete areas of the blastoderm being displaced inwards via the primitive streak. This results in the formation of the three embryonic germ layers; ectoderm, mesoderm and endoderm. The initial inward movement of cells from the surface layer or epiblast of the blastoderm results in the displacement of cells of the underlying hypoblast layer to the peripheral regions of the blastoderm. The incoming cells form the embryonic endoderm, whereas peripheral hypoblast cells form the extraembryonic endoderm. Some of the ingressing cells

form the mesodermal layer which lies between the surface ectoderm and the lower endoderm (Romanoff, 1960; Bellairs, 1971; Nicolet, 1971).

1.2 Formation and Structure of the Yolk Sac

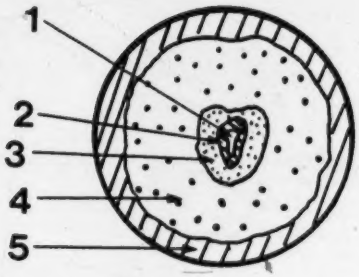
At the primitive streak stage (18 hours of incubation), the chick blastoderm consists of two areas; the area pellucida, consisting of ectoderm, mesoderm and endoderm, which will form the embryo, and the extraembryonic area opaca, which is made up of ectoderm and endoderm, the latter tissue forming a permanent part of the yolk sac. Within two days of development, the mesoderm begins to invade the area opaca or presumptive yolk sac membrane and blood islands are evident. At this time, the area opaca consists of two distinct areas, the area vasculosa, a medial vascularized region adjacent to the embryo and the area vitellina, a more peripheral region that is non-vascularized (Figure 1A). The area opaca can be termed the yolk sac membrane at this time, since the horizontal division of mesoderm into somatic and splanchnic layers is apparent and the splanchnic mesoderm and endoderm have associated to form the yolk sac (Romanoff, 1960).

The morphogenesis of the yolk sac involves three expanding cell layers, the ectoderm and endoderm of the vitelline area and the mesoderm of the vascular area. The peripheral margin of the area vitellina is the leading edge of the yolk sac which progresses over the yolk surface very rapidly between the third and fifth days of incubation (Figure 1B-D). The yolk is usually completely surrounded by the end of the seventh day of incubation, except for a small aperture at the distal end, called the yolk sac umbilicus (Figure 1E). Despite the continued peripheral extension of the area vitellina, it begins to decrease in total area by

Figure 1

Schematic illustrations of successive stages in the growth of the yolk sac over the surface of the yolk, during the first seven days of chick development (redrawn from diagrams by Romanoff, 1960). A and A1, dorsal and lateral views of a chicken embryo at 2 days of development; B and B1, dorsal and lateral views of a chicken embryo at 3 days of development; C and C1, dorsal and lateral views of a chicken embryo at 4 days of development; D, dorsal view of a chicken embryo at 5 days of development; E, lateral view of a chicken embryo at 7 days of development; 1, embryo; 2, area pellucida; 3, area vasculosa; 4, area vitellina; 5, yolk; 6, sinus terminalis; 7, amnion; 8, allantois; 9, yolk sac umbilicus.

A



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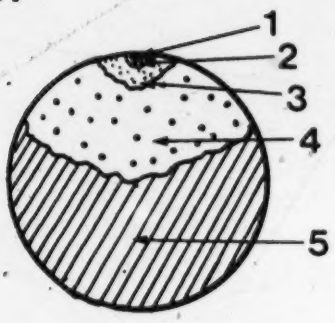

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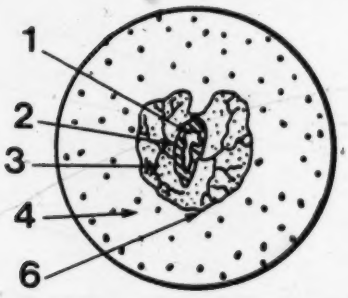

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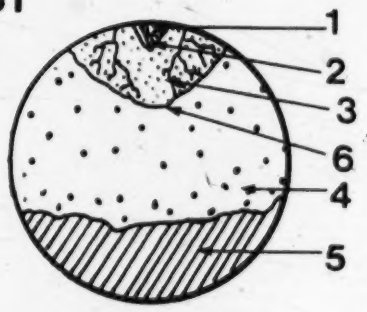

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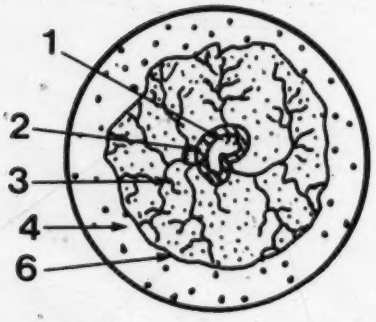

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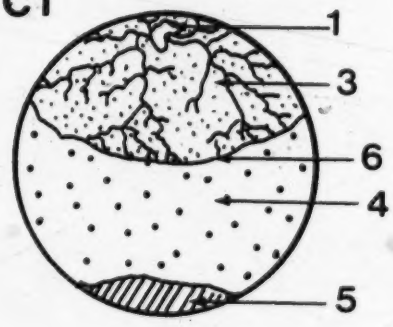

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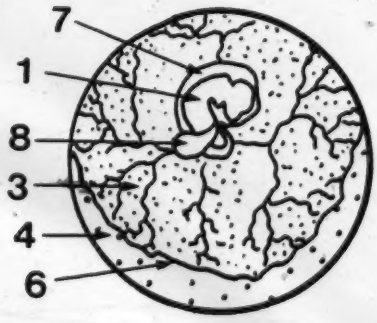

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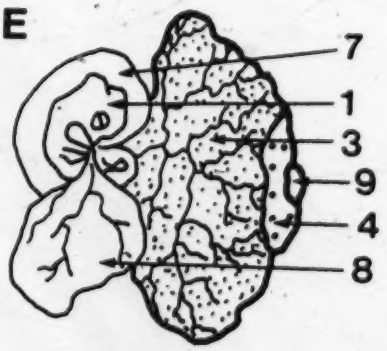


the seventh day because of encroachment by the area vasculosa. This latter tissue also grows very rapidly between the third and fifth day of incubation and has passed the equator of the yolk between five and seven days of incubation. The area vasculosa continues to grow until the fourteenth or fifteenth day and it reaches the margin of the yolk sac umbilicus between sixteen and nineteen days of incubation, at which time this aperture closes; by this time the area vitellina has disappeared completely. The yolk sac is taken up into the embryo's body cavity via the yolk stalk, which is a peduncle that attaches the yolk sac to the intestine. This occurs on or after the nineteenth day of development. The embryo hatches on the twenty-first day after the start of incubation (Romanoff, 1960).

1.3 The Extraembryonic Endoderm

The yolk sac is in a continuous state of change throughout development. Its constituent germ layers are different at the beginning and end of the formative period. As mentioned previously, the endoderm forms a permanent part of the yolk sac. At the primitive streak stage, these cells are present as a multilayered array in the ventral surface of the area opaca which lies adjacent to the yolk. The yolk sac endoderm goes through several developmental stages as it spreads by epiboly to surround the yolk and finally differentiates into a single cell epithelial layer (Romanoff, 1960; Bellairs, 1963, Mobbs and McMillan, 1979). The successive stages in this process can be seen in the structure of these cells from the peripheral to the proximal parts of the yolk sac, as it grows. The most immature stages are found at the

periphery of the area vitellina where new cells are formed. In this area nuclei are scattered sparsely in the yolk and no cell boundaries are distinguishable. The more mature or fully differentiated stages are found medially in the area vasculosa region, where a single layered epithelium is present. Transitional stages in the development of the endoderm are found between these two regions, where there is a zone of irregularly stratified cells composed of three to four layers (Romanoff, 1960).

By the end of the second day of incubation, the medial stratified zone of cells has moved distal to the area vasculosa and is part of the inner region of the area vitellina. This stratified zone spreads centrifugally by the addition of cells from the peripheral zone, while cells at its medial border differentiate as columnar epithelial cells and join the epithelial zone, which is present below the area vasculosa. The stratified zone of cells is completely transformed into epithelium at some time after the twelfth day of incubation (Romanoff, 1960).

2. THE CELL SURFACE

A widely held view contends that glycoproteins and glycolipids on the surface of cells are implicated in the processes of cellular recognition and adhesion. The cell surface is the site of a cell's interaction with other cells as well as its extracellular environment in both embryonic development and in adult tissues (Cook and Stoddart, 1973; Hughes, 1975; Lackie, 1980).

Cell surface carbohydrate consists of short oligosaccharide chains of membrane bound glycoproteins and glycolipids. In addition, it often

includes both glycoproteins and proteoglycans that have been secreted and absorbed on the cell surface (Hughes, 1976; Luft, 1976). Isolated plasma membranes typically contain 2 to 10 percent carbohydrate localized on the outer surface. Nine monosaccharides generally occur in membrane glycoproteins and glyocolipids, the principal ones being galactose, mannose, fucose, galactosamine, glucosamine, glucose and sialic acid. The sialic acid residues are usually located on the terminus of the carbohydrate side chain and contribute, for the most part, to the net negative surface charge of eukaryotic cells.

Although the oligosaccharide side chains of glycoproteins and glycolipids contain fewer than fifteen sugar residues they are usually branched and joined by a variety of different linkages which can confer great complexity. There can be considerable diversity in the saccharide side chains and the variation from one cell type and species to another can give the cell a unique identity, with carbohydrate moieties possibly serving as discrete sources of information or recognition (Hughes, 1975).

The biological function of the carbohydrate moieties of glycoproteins has not been fully eludicated. However, the wide distribution of these macromolecules and the conserved structure of the oligosaccharide units suggests that they are important in some universal physiological process in animal cells (Olden et al., 1982). Roth et al. (1971) have suggested that specific intercellular adhesion could be mediated by the interaction of carbohydrate moieties of membrane or extracellular protein on the surface of one cell with a carbohydrate-binding protein, a glycosyltransferase, on another cell surface. The

complexity of oligosaccharides and their position at the cell surface makes them suitable candidates for recognition and/or adhesion.

Olden et al. (1982) have proposed that the covalently attached carbohydrate of glycoproteins acts as a 'chemical tag' which directs the glycoprotein to certain locations or organelles within the cell, through its interactions with specific intracellular membrane receptors. In this proposal, glycosylation is not absolutely necessary for secretion of glycoprotein nor is it always required for the biological activity of the protein. But it does appear to be involved in the proteolytic stabilization of the protein molecule. Evidence to support this proposal comes from work done with the antibiotic, tunicamycin, which inhibits the glycosylation of asparagine-linked glycoproteins and with mutants that are deficient in glycosyltransferases (reviewed by Olden et al., 1982). These authors present a model in which the carbohydrate moiety functions in the localization of glycoprotein intracellularly through its interaction with endogenous carbohydrate binding protein. Such proteins located on the luminal surface of the Golgi-endoplasmic reticulum complex, bind and concentrate the appropriate oligosaccharide moiety in specific areas of this internal membrane system and then transports them via vesicles to specific membrane organelles, including the cell surface.

3. LECTINS

3.1 Definition and Historical Background

Lectins are a class of divalent or multivalent carbohydrate-binding

grouped together because of their ability to precipitate glycoconjugates and/or agglutinate cells. Lectins bind to cell surfaces through their interactions with specific oligosaccharide determinants exposed on the surface of cells. The sugar-binding specificity of these proteins is normally defined in terms of the monosaccharides or simple oligosaccharides that inhibit lectin induced agglutination of cells or lectin induced precipitation of polysaccharides, glycoproteins and glycolipids (Goldstein et al., 1980).

Proteins able to agglutinate animal erythrocytes were first identified in plant seed extracts. They were studied intensively because they have a number of unusual chemical and biological properties which make them useful biological probes in research. In particular is their specificity for particular sugar residues and their divalency or polyvalency (Lis and Sharon, 1973). These agglutinins were first called lectins (from the Latin, legere, to pick out or to choose) by Boyd and Shapleigh (1954) after the discovery that some plant agglutinins are specific for certain human blood groups. For example, the lima bean agglutinin was shown to selectively agglutinate human type A erythrocytes and not type B or type O (Reviewed by Boyd, 1970).

Two important discoveries in the 1960's set the pace in plant lectin research. In 1960, Nowell described the ability of phytohaemagglutinin (PHA), from the red kidney bean (Phaseolus vulgaris), to transform small resting lymphocytes into large, actively growing blast-like cells which sometimes divide. Subsequently in 1963, Aub et al. reported that wheat germ agglutinin (WGA), from Triticum

vulgare, preferentially agglutinated malignant cells at low concentrations, while higher concentrations of this lectin were necessary for agglutination of normal cells. Cells transformed by mitogenic lectins, such as PHA, have been used in the visual examination of chromosomes in humans and other animals. Also, the stimulation of lymphocytes by these lectins has facilitated the study of mechanisms by which signals are transmitted from the surface of the cell to the nucleus and the metabolic machinery of the cytoplasm, and the examination of the biochemical events involved in the activation of a resting cell. Lectins have also been used for studying changes at the cell surface during growth, differentiation and malignant transformation. Other important uses of plant lectins in biological research included their use in typing human blood and in the study of the structures of the blood group antigens (Sharon and Lis, 1972; Lis and Sharon, 1973, 1981; Nicolson, 1974; Goldstein and Hayes, 1978).

3.2 Detection and Carbohydrate Specificity

As mentioned previously, the initial identification of plant lectins was by their ability to agglutinate animal erythrocytes and this is still the common method of choice for detecting the presence of lectins (Goldstein and Hayes, 1978; Lis and Sharon, 1981). This property of lectins suggests that these proteins are at least divalent and possibly multivalent with regard to their carbohydrate-binding sites and serves as the basis for their detection and quantitation.

The agglutination reaction is not well understood and it can be influenced by a variety of factors including properties of the lectin molecule such as its biochemical nature, the number of saccharide-

binding sites it contains, the strength of saccharide binding and the concentration of lectin activity in the extracts. Properties of the cell surface receptors such as the number, accessibility, distribution and mobility of receptor sites also affect the agglutination reaction. In addition general cell surface structures such as microvilli, cell surface rigidity, net cell charge and transmembrane linkages of receptors to components of the cytoskeleton may also affect this reaction (Nicolson, 1974). Despite the lack of information on this reaction, agglutination assays are used because they are a rapid and convenient method for the detection and quantitation of lectin activity.

The saccharide-binding specificity of a lectin is usually determined by the ability of specific sugars to inhibit lectin mediated agglutination. And for some time it was generally accepted that the lectin bound exclusively to terminal sugar groups and that the best inhibitor was similar to the cell surface receptor. However it has since been shown that some plant lectins can interact with sugar groups at core sites in oligosaccharides, in addition to interacting with terminal residues. For example, concanavalin A (Con A), can bind to terminal α-D-mannose or α-D-glucose residues or internal α-D-mannose residues (Goldstein et al., 1973). And it has also been shown that the interactions of lectins with complex synthetic oligosaccharides or with oligosaccharides isolated from cell surfaces are stronger than with simple sugars. This suggests that the actual cell surface lectin receptors are most probably complex oligosaccharides (Nicolson, 1974).

While there are limitations to the saccharide inhibition assay some information on the lectins carbohydrate-binding specificity can be obtained from such studies. Alkyl α- and β-glycosides are often used as hapten inhibitors to give information on the anomeric specificity of a lectin (Lis and Sharon, 1981). Some lectins have been found to be specific for only one anomeric configuration, whereas others do not appear to distinguish between the two. Con A is an example of the former situation with specificities for α-D-mannose and α-D-glucose only. Ricinus communis agglutinin (RCA), and soybean agglutinin (SBA), on the other hand, do not appear to exhibit anomeric specificity (Lis and Sharon, 1981).

From studies with plant lectins (reviewed by Lis and Sharon, 1981) it appears that the carbon-2 position of the sugar is not of crucial importance to the interaction for most plant lectins. Thus lectins such as Con A, which exhibit a primary specificity for α-D-mannose also bind α-D-glucose and N-acetyl-D-glucosamine to a lesser extent. Conversely, several lectins have a primary specificity for galactose and also bind N-acetyl-D-galactosamine to some extent. However, the carbon-4 position of the sugar appears to be critical to lectin binding. Thus lectins which bind galactose do not normally interact with mannose or glucose and vice-versa.

The sugar-lectin binding reaction appears to involve hydrogen bonds and nonpolar interactions. Because most sugars are not charged, the interaction is not thought to involve electrostatic forces (Lis and Sharon, 1981). Higher affinity constants are generally observed for lectin binding to cell surface carbohydrates, as compared to simple

sugars. And it has been assumed that the interactions between a lectin molecule and cell surface carbohydrate is through multivalent interactions involving more than one combining site (Lis and Sharon, 1981). Further, the cell surface glycoconjugates are thought to probably be functionally multivalent, since univalent glycoconjugates can move and form clusters within the plane of the membrane. Thus lectins can bind well to these clusters although they may not interact with high affinity to the individual carbohydrate molecules (Barondes, 1983).

3.3 Endogenous Lectins

Following the detection of lectins in plant tissues, similar carbohydrate-binding proteins were isolated from a wide variety of microorganisms, invertebrate and vertebrate tissues. Because endogenous carbohydrate-binding proteins or lectins possess divalent or multivalent binding capacities and are carbohydrate specific, these macromolecules are obvious candidates for the binding of specific oligosaccharide determinants on a cell's surface and may play a part in the processes of recognition and/or adhesion. Studies so far indicate that endogenous lectins are predominantly intracellular but are also found on the cell surface or extracellularly. In a recent review by Barondes (1981), several properties of lectins that suggest biological functions were evaluated. They include the following: a) the specificity of the saccharide-binding sites of lectins suggests that endogenous receptors are present, either on the cells which contain the lectin or on other cells or glycoconjugates with which the lectin is to interact; b) the

possession of multiple carbohydrate-binding sites suggests that the lectin could organize glycoconjugates on the cell surface and extracellularly, by cross-linking glycoproteins or glycolipids; c) the agglutination activity of lectins suggests they could function by binding together cells of the same or different species, thus promoting adhesion in the morphogenesis of embryos or tissues, or promoting phagocytosis or infection; d) the high levels of lectin activity generally found indicates a structural role for lectins, as opposed to an enzymatic function; and e) the finding of lectin activity in soluble aqueous extracts of tissues suggests lectins are not generally integrated into membranes but are probably bound to membranes by oligosaccharide receptors.

Some experimental approaches to identifying the function of lectins include: a) determining the localization and distribution of lectin in tissues by histochemical methods, using labelled haptens and antibodies; b) determining lectin concentration and changes in levels of activity in developmental studies; c) studies of mutants that possess abnormal lectin or which are deficient in lectin; d) studies on in vitro and in vivo biological effects of lectin antagonists such as antibodies to lectin and haptens that inhibit lectin; and e) the identification and purification of possible endogenous receptors (Barondes, 1981).

Vertebrate endogenous lectins have been divided into two groups on the basis of their solubility properties (Barondes, 1984). Soluble lectins do not require detergents for their extraction and can be extracted from tissues by specific saccharides which remove them from their endogenous ligands. These lectins are probably loosely

associated, peripheral membrane proteins and are believed to be able to move freely within and between cells. They would thus be able to interact with both soluble and membrane bound glycoconjugates. Because of these properties, a function for soluble lectins has been difficult to infer. Integral membrane lectins, on the other hand, require detergent for extraction and are thought to function in the binding of glycoconjugates to membranes at the cell surface or within vesicles. This mechanism would result in the localization of specific glycoconjugates at particular membrane sites or their transport to other cellular compartments (Neufeld and Ashwell, 1980; Barondes, 1984).

Information on the molecular mechanisms that mediate cell recognition and adhesion has been obtained from studies on sponges and cellular slime molds. These two systems are discussed below because they have contributed to an understanding of these cell surface phenomena.

3.3.A. The Sponge Lectins

A number of lectins have been identified in sponges, the majority of which appear to be galactose-binding proteins. These lectins can be isolated and purified from the supernatant in which the sponge cells are dissociated (Müller, 1982). In the sponge Geodia cydonium, a major β-D-galactoside-binding lectin (MW 36,500) has been isolated from tissue extracts (Vaith et al., 1979; Müller et al., 1983). In addition, three other adhesion related components including an aggregation factor (Müller and Zahn, 1973), an aggregation receptor (Müller at al., 1976) and an anti-aggregation receptor (Müller et al., 1979a) have been

isolated from this sponge. In aggregation assays, the anti-aggregation receptor inhibits aggregation factor-receptor formation by binding to the aggregation factor. This effect can be abolished by pretreating the anti-aggregation receptor with β-galactosidase, which suggests that terminal galactose is the active site of this molecule (Müller et al., 1979a). The galactose-binding lectin precipitates the anti-aggregation receptor and abolishes the inhibition of aggregation caused by this glycoprotein. Thus the lectin does not appear to have a direct role in promoting aggregation. But it does appear to play a regulatory role and could, in principle, control the processes of sorting out and recognition in aggregation, by allowing transitory adhesion or deadhesion to occur (Müller, 1972; Müller et al, 1979b; Müller and Müller, 1980).

3.3.B. Lectins in the Slime Mold Developing System

A function for endogenous lectins as recognition molecules in development has been extensively investigated using cellular slime molds. The hypothesis is that species-specific cell cohesion results from the complementary interaction of cell surface endogenous lectins and cell surface carbohydrate receptors to which the lectin binds specifically (Bartles et al., 1982). These simple eukaryotes have served as a good model for studies of intercellular recognition and adhesion. In their unique life cycle they exist as nonsocial, single, vegetative amoebae and only differentiate to an aggregation competent stage when deprived of food. During this stage of development cells are drawn together by chemotactic signals, specific cell contacts are made and a stable, social aggregate called a plasmodium is formed. Further

development results in formation of a fruiting body made up of stalk and spore cells (Frazier, 1976).

Agglutinating activity has been detected in soluble extracts of cells of Dictyostelium discoideum (Rosen et al., 1973) and Polysphondylium pallidum (Rosen et al., 1974) that are aggregation competent. Since this activity is absent from non-cohesive, vegetative cells, it is thought to be developmentally regulated. Similar activity has been found in all species of slime mold so far studied (Rosen et al., 1975). Two distinct lectins with slightly different carbohydrate-binding specificities and subunit molecular weights were isolated from D. discoideum. Discoidin 1 (MW 26,000) is specific for N-acetyl-D-galactosamine and discoidin II (MW 24,000) has a specificity for D-fucose (Simpson et al., 1974; Frazier et al, 1975). Pallidin, the lectin from P. pallidum has a subunit molecular weight of about 25,000 and is specifically inhibited by lactose (Simpson et al., 1975; Rosen et al., 1979). These lectins are tetrameric and can be further distinguished by their isoelectric points and amino acid compositions. Both discoidin I and pallidin appear to exist in multiple forms or as isolectins with differing isoelectric points and electrophoretic mobilities. In both species, the lectins appear to be present on the cell surface of aggregation competent cells. This has been demonstrated by the ability of these cells to bind enythrocytes in the presence of non-specific sugars which do not interact with lectin, but not in the presence of lectin-specific sugars and by immunofluorescent and immunoferritin labelling studies (Chang et al., 1975, 1977; Siu et al., 1976).

Some evidence for a role in adhesion for these lectins has been obtained from studies showing that certain factors that inhibit haemagglutination activity also inhibit intercellular adhesion of aggregation competent cells. Certain potent macromolecular antagonists of pallidin, including asialofetuin, a glycoprotein with terminal galactose residues and specific univalent anti-pallidin antibody, inhibited aggregation of P. pallidum cells under 'permissive conditions,' in which cells were exposed to hypertonic conditions or to antimetabolites (Rosen et al., 1977). These workers suggest that pallidin is directly involved in the developing cohesiveness of these cells because competitive inhibition of the lectin would be expected to interfere with cell adhesion, if such lectins were to mediate cell-cell adhesion by binding to oligosaccharide receptors at the cell surface.

Further evidence used to support a role for these endogenous slime mold lectins in species-specific, cell-cell recognition and adhesion during development comes from studies with mutant slime molds (Ray et al., 1979; Shinnick and Lerner, 1980). The mutants appear to synthesize abnormal or functionally impaired lectin and exhibit reduced adhesion and aborted development. In addition, developmentally regulated species-specific receptors for the slime mold lectins have been demonstrated on the surface of differentiating cells (Reitherman et al., 1975; Chang et al., 1977; Breuer and Siu, 1981; Drake and Rosen, 1982). These high affinity receptors are thought to be either extracellular or integral cell surface glycoconjugates. This has been shown by several studies, including cell agglutination by purified lectins and binding measurements in which lectin binding to cells was quantified.

Recent work on the discoidin lectins shows that synthesis of these proteins is regulated differently (Ma and Firtel, 1978; Cooper and Barondes, 1984). A spore coat polysaccharide that appears to be an endogenous receptor for discoidin II, is synthesized and secreted from prespore cells late in development. This macromolecule is composed of mainly galactose and N-acetyl-D-galactosamine and binds well to the lectin in competitive binding assays (Cooper et al., 1983). The lectin and receptor appear to interact with each other both inside and outside the prespore cell, but the lectin is absent from mature spores (Barondes et al., 1983; Cooper and Barondes, 1984). A macromolecule in the slime coat of early aggregates appears to be the endogenous receptor for discoidin I (Cooper et al., 1983; Cooper and Barondes, 1984). This lectin appears to be synthesized during aggregation and is secreted into the slime coat of maturing aggregates. Barondes et al. (1983) have suggested that the lectin functions in organizing the extracellular material around the aggregate or in slime trails. And it was subsequently shown that discoidin I acts like fibronectin, a cell attachment molecule, with which it has amino acid sequences in common; it appears to promote cell attachment and spreading, as well as ordered migration of cells during morphogenesis (Springer et al., 1984).

The hypothesis that slime mold lectins are involved in cellular recognition and adhesion has been questioned recently, since evidence for such a role is inconclusive and since other cell surface glycoconjugates appear to be involved in adhesion (Bartles et al., 1982). The major argument against a direct role for lectins comes from

the inconclusive and negative results of cell adhesion experiments in which competitive inhibitors of the lectin are used. The results reported by Rosen et al. (1977) for the effect of univalent anti-pallidin antibody were not observed under standard physiological conditions. And Springer and Barondes (1980) were unable to obtain inhibition of adhesion of D. discoideum cells with similar univalent antibodies. On the other hand, univalent antibody against the cell surface glycoproteins, contact site A, gp 150 and gp 95 which have been implicated in cell adhesion, do inhibit adhesion. These results have led to the speculation that the lectins may have a regulatory role, rather than a direct role in intercellular adhesion. This regulation may be of the expression or activity of the glycoproteins gp 150, gp 95 and contact site A (Bartles et al., 1982).

Bartles et al. (1982) have also suggested a possible alternate role for the slime mold lectins in intracellular functions. This is based on the predominantly intracellular localization of these lectins. For example, Springer et al. (1980) determined that up to 98 percent of lectin in D. discoideum is obtained in soluble form following cell lysis. At least a part of the intracellular lectin seems to be bound to intracellular receptors, because higher yields of lectin are obtained when cell lysis is performed in the presence of hapten sugars or high ionic strength buffers. Almost no additional lectin could be subsequently extracted with non-ionic detergents (Springer et al., 1980). Barondes (1984) has also suggested an alternate role for slime mold lectins based on the recent studies on discoidin lectins reviewed earlier. The lectin-receptor interactions may function in formation of

large macromolecular complexes that shape the extracellular environment, which is important in morphogenetic processes.

3.3.C. The Mammalian Hepatic Lectin

In 1974, Stockert and coworkers isolated and characterized what was believed to be the first lectin of mammalian origin. It was a cell surface glycoprotein purified from extracts of rabbit liver membranes (Hudgin et al., 1974). This lectin binds specifically to asialoglycoproteins and it also agglutinates untreated human and rabbit erythrocytes as well as neuraminidase treated erythrocytes from guinea pig, rat and mouse. Both of these effects appear to involve reaction at the same active site of the protein (Stockert et al., 1974). This hepatic binding protein functions in carbohydrate and glycoprotein metabolism by the specific recognition, binding and uptake of desialylated glycoproteins from the blood.

In a series of experiments, (reviewed by Ashwell and Morell, 1974; Neufeld and Ashwell, 1980) it was shown that the carbohydrate moiety of certain serum glycoproteins functions in their survival or clearance from the blood. The hypothesis advanced by these workers was that sialic acid residues have to be present on the glycoprotein for it to remain in the circulation. The degradation of terminal sialic acid residues causes the immediate clearance of the desialylated molecules by the liver. The specific recognition of desialylated glycoprotein thus depends on the presence of non-reducing terminal β-galactosyl groups which are exposed on removal of sialic acid. The key role for galactose in the recognition event was demonstrated by treating the protein,

bearing the exposed galactose groups, with galactose oxidase or β-galactosidase, which restored the native survival time of the serum glycoprotein molecule (Morrell et al., 1968). In addition, restoration of sialic acid residues by the action of sialyltransferase prolonged the survival time of the glycoprotein (Hickman et al., 1970). This observation further supported the idea that terminal galactose residues are recognized. The binding and uptake of desialylated glycoproteins also requires the presence of intact sialic acid residues on the hepatic binding glycoprotein membrane receptor, as well as the presence of calcium (Pricer and Ashwell, 1971; Hudgin et al., 1974; Stockert et al., 1977).

3.3.D. The Electric Eel Lectin

Teichberg and coworkers (1975), described a β-galactoside binding protein isolated from extracts of a variety of mammalian tissues and tissue culture cells. They specifically purified and characterized this protein from the electric organ of the eel, Electrophorus electricus and called it electrolectin. They showed that the lectin agglutinates trypsin treated rabbit erythrocytes and is specifically inhibited by saccharides containing non-reducing, terminal β-galactose residues, such as thiodigalactoside and lactose. This lectin also requires the presence of a reducing agent to maintain agglutination activity. The protein which has a subunit molecular weight of about 16,500, is a dimer which appears to be stabilized by the presence of lactose (Levi and Teichberg, 1981). These workers did not suggest a function for this lectin.

However, the work done by Teichberg and coworkers stimulated a great deal of work on other vertebrate tissue lectins. In particular is an interest in cellular differentiation and the possible mechanistic role for lectins during the development of tissues. The term, developmentally regulated lectin, first used with slime mold lectins, is used here for lectins that are shown to be particularly prominent at a specific stage in the development of the tissue. A developmentally regulated lectin plays a specific role and is required at a specific time and then diminishes in importance after the differentiative process has ended (Barondes, 1981).

3.3.E. The Embryonic Chicken Pectoral Muscle Lectin

Recent studies of the role of lectin(s) in development have focused on muscle because this tissue has been widely used in in vitro studies of vertebrate cellular differentiation. Nowak et al. (1976), found that soluble extracts from embryonic chick pectoral muscle and a myogenic cell line derived from rat skeletal muscle could agglutinate trypsin treated, glutaraldehyde fixed rabbit enythrocytes. This agglutination activity could be specifically inhibited by thiodigalactoside and lactose and varied with the stage of development, suggesting a developmentally regulated lectin(s). Lectin was present at low levels during early stages of development and increased with muscle differentiation, reaching its highest levels at 16 days of development. Lectin levels then declined to very low levels in adult tissue. A similar increase with development was found in differentiating L6 myoblasts in culture. The muscle lectin has been purified by affinity chromatography and is a dimer with a subunit molecular weight of about

15,000 (Den and Malinzak, 1977; Nowak et al., 1977). Immunofluorescent techniques have been used to demonstrate the presence of lectin on the surface of cultured myoblasts, however the predominant location of the lectin was intracellular (Nowak et al., 1977; Podleski and Greenberg, 1980).

The role of this developmentally regulated lectin in myoblast fusion is controversial. The presence of high concentrations of lectin during myoblast fusion, its extracellular location and the inhibition of myoblast fusion by thiodigalactoside has been used as support of its role in early muscle development (Gartner and Podleski, 1975, 1976). However, thiodigalactoside was subsequently found to have no effect on fusion of chick myoblasts in culture (Den et al., 1976), which conflicts with the results reported by Gartner and Podleski (1975, 1976). Other recent studies report that repeated addition of purified chick muscle lectin to embryonic chick myoblasts in culture inhibits myotube formation (MacBride and Przybylski, 1980). However since various plant lectins also impair muscle fusion, these results have been difficult to interpret (Barondes, 1981). Recent work by Barondes and Haywood Reid (1981) indicates that the muscle lectin, which is concentrated intracellularly in developing muscle, is secreted into the extracellular matrix by polynucleated myotubes. This has been interpreted as evidence against a role for the lectin in the earlier process of myoblast fusion. The apparent secretion of lectin into extracellular matrix suggested a function in binding glycoconjugates at this location. Thus at the

present time no specific role for the soluble β-galactoside-binding lectin in myoblast fusion has been established.

3.3.F. Soluble Lectins from Vertebrate Embryonic and Adult Tissues

The finding of similar soluble, dimeric β-galactoside-binding lectins in extracts of a variety of embryonic chicken tissues including brain, heart and liver (Kobiler and Barondes, 1977; Kobiler et al., 1978) and adult chicken tissues including liver, pancreas and intestine (Beyer et al., 1979, 1980; Beyer and Barondes, 1982a) suggests that lectins are not tissue specific. The lectin purified from adult chicken liver appears to be immunologically identical to that from embryonic skeletal muscle (Beyer et al., 1980). Thus it appears that this lectin may have different roles in different tissues and in addition to a developmental function in embryonic tissue, it may have a different role in adult tissues of the same organism (Barondes, 1981).

Chicken lactose lectin I (CLL-I) is a dimer and can be distinguished from a different galactose-binding lectin chicken lactose lectin II (CLL-II) which is a monomer (Barondes, 1981, 1984). The latter is also developmentally regulated and has been purified from adult chicken intestine (Beyer et al., 1980). It is found at very high levels at this location, but is present at only low levels in the embryo. It is also found in embryonic liver and kidney where it is very prominent, but is scarce in adult liver and kidney as well as in embryonic muscle and brain (Beyer and Barondes, 1982a). CLL-II differs from the dimeric CLL-I, in subunit molecular weight, isoelectric point, peptide map and it is also immunologically distinct (Beyer et al., 1980). Because CLL-II is a monomer and can agglutinate erythrocytes it

is thought to either have two carbohydrate-binding sites per monomer or to be able to dimerize to some extent (Barondes, 1984). Although a function for this lectin in development has not been elucidated, a possible role in the secretory function of intestinal goblet cells has been suggested (Beyer and Barondes, 1982b). CLL-II has been immunohistochemically localized in the secretory granules of these cells and on the mucosal surface of intestine. And intestinal mucin, which is a highly glycosylated glycoprotein containing many terminal β-galactoside residues, is also localized in the same secretory granules and it appears to be an endogenous ligand for CLL-II. The mucin and lectin bind well to each other and it has been suggested that lectin-glycoconjugate interactions in the secretory granules may block mucin-mucin interactions prior to secretion (Barondes, 1984). Upon release, the individual mucin molecules may associate through carbohydrate-carbohydrate interactions to form complex structures that ultimately exclude the lectin.

Similar lectins have since been isolated from many different vertebrate species. These soluble β-galactoside-binding lectins are similar to CLL-I. They are dimeric proteins, with subunit molecular weights in the range 10,000-16,500 and they have a requirement for a reducing agent to maintain carbohydrate-binding activity. Such lectins have been identified in a variety of adult bovine tissue including heart and lung (DeWaard et al., 1976), thymus, liver and spleen (Briles et al., 1979). These lectins are indistinguishable by physical, immunological and carbohydrate-binding properties.

Rat tissues also contain a dimeric, β-galactoside-binding lectin which has been isolated from lung tissue, where it reaches a peak of activity during development (Powell, 1980; Powell and Whitney, 1980), and from skeletal muscle (Gartner and Podleski, 1975, 1976). In lung, this lectin is present intracellularly, as well as extracellularly in all the elastic fibers of this tissue, including those of the lung parenchyma and blood vessels, and thus may have a function in tissue elasticity (Cerra et al., 1984). Another lectin found in rat lung tissue reacts with heparin and related glycosaminoglycans (Roberson et al., 1981), and a similar heparin-binding lectin has been detected in embryonic chicken muscle and adult liver (Ceri et al., 1979; 1981). These lectins may play a role in the association of extracellular glycosaminoglycans with the cell surface or in the organization of extracellular glycoconjugates. β-galactoside-binding lectins have also been isolated from human tissues, including lung (Childs and Feizi, 1979; Powell, 1980).

3.3.G. Lectins in Embryonic Development

A major problem in developmental biology is elucidation of the mechanism(s) by which embryonic cells migrate and orient themselves in specific organized ways during the complex morphogenetic movements of gastrulation. These movements and cell rearrangements result from cell-cell and cell-extracellular matrix interactions involving formation of specific intercellular contacts which would suggest that the cells are able to recognize and distinguish between each other.

A number of studies have shown that embryonic cells exhibit selective cellular affinities indicating that cellular recognition

occurs (Holtfreter, 1939; Townes and Holtfreter, 1955; Moscona, 1974). Glycoproteins on the surface of embryonic cells are assumed to play important roles in early embryogenesis and their presence at this site has been shown by a number of methods, including the use of plant lectins (Nicolson, 1974; Oppenheimer, 1977). Specifically, these lectins have been used as probes to investigate the nature and functional significance of these surface glycoconjugates and in studying cell surface changes during development, in a number of embryonic systems, including the sea urchin embryo (Krach et al., 1974; Roberson et al., 1975), the amphibian embryo (O'Dell et al., 1974; Moran, 1974; Johnson and Smith, 1976; Fraser and Zalik, 1977) and the chicken embryo (Zalik and Cook, 1974, 1976; Phillips and Zalik, 1982).

Following the identification of carbohydrate moieties on the surface of embryonic cells, a search was made for endogenous lectins which could bind to these surface carbohydrate receptors and could be involved in the processes of cell recognition and adhesion during embryogenesis. Such endogenous lectins have been identified in amphibians, specifically in the oocytes and early embryos of Xenopus laevis (Harris and Zalik, 1982; Roberson and Barondes, 1982). Roberson and Barondes (1982) have described a soluble, galactoside-binding lectin purified from blastula stage embryos and oocytes, which appears to be a glycoprotein with a native molecular weight, in solution, of about 480,000 and appears to be an aggregate of two subunits with molecular weights of about 43,000 and 45,000. It requires calcium for agglutination activity and shows heterogeneity on isoelectric focusing.

Roberson and Barondes subsequently (1983) demonstrated immunohistochemically that the lectin was associated with yolk platelets, cortical granules and the vitelline envelope of oocytes and unfertilized eggs. It was also localized extracellularly in the cleavage furrows between cells of blastula-stage embryos. The most frequent location was in the yolk platelets where it was found near the surface, but the way in which the lectin is associated with these structures is not yet known. The lectin is thought to be in storage form at this location and it may be externalized to cleavage furrows, where it might function by binding to glycoconjugates in the extracellular matrix. These workers concluded that the lectin may have multiple functions in this developing system, one of which may be a role in organizing the extracellular matrix.

Harris and Zalik (1982) detected an apparently identical soluble galactoside-binding lectin in Xenopus laevis embryos of the same and later developmental stages, including gastrula and neurula. Subsequent work (Harris and Zalik, 1985) indicates that the lectin is developmentally regulated with highest levels of activity in gastrula stage embryos. The purified lectin which was treated with chloroform/methanol has an approximate molecular weight of 65,500 and was shown to be an aggregate with a molecular weight of about 371,000 in aqueous solution. The chloroform/methanol treatment used in this study removed material in the molecular weight range of 37,700 to 48,000, which corresponds to the molecular weight for the lectin reported by Roberson and Barondes (1982). This material is thought to be a glycolipoprotein that associates tightly with the lectin during extraction and influences

the aggregation of the lectin molecule. These workers have still to determine where the lectin is localized and if it is found at the same sites as those reported by Roberson and Barondes (1983). A function for the endogenous lectin in cell-cell interactions during gastrulation has been suggested (Harris and Zalik, 1982, 1985).

Cells of the gastrulating chicken embryo have also been shown to contain agglutinable surface receptors bearing terminal galactosyl groups (Zalik and Cook, 1976; Phillips and Zalik, 1982). The presence of such glycoprotein receptors at the surface of these embryonic cells suggests that endogenous carbohydrate-binding proteins may also be present.

Cook and coworkers (1979) have shown that extracts of pregastrula and gastrula stage embryos can agglutinate trypsin treated, glutaraldehyde fixed rabbit erythrocytes. This agglutination could be inhibited by saccharides bearing a β-D-galactoside configuration, suggesting that the endogenous lectin binds to these groups. Lectin activity was found in both embryonic and extraembryonic areas of the blastoderm. In further work, Zalik et al. (1981, 1983) purified and characterized the two β-D-galactoside-binding lectins. One of them is an easily extracted, soluble lectin with an approximate subunit molecular weight of 11,000. This lectin is found in both the embryonic area pellucida and the ectoderm and endoderm of the extraembryonic area opaca. The second lectin is a particle-associated molecule requiring longer extraction periods, with subunit molecular weights of about

70,000 and 72,000. This lectin is found at high concentrations in the area opaca, particularly in the endodermal cells of this region.

Milos and Zalik (1982) have suggested that the endogenous β-D-galactoside-binding lectins could mediate a surface related function by combining with glycoprotein receptors bearing terminal galactose groups. Cells isolated from the extraembryonic endoderm of gastrulating embryos and cultured in rotating flasks show decreased cellular adhesion that is associated with increased levels of lectin activity in the medium. This activity is believed to be released into the medium by the cells. It was also shown that adding exogenous blastoderm lectin or thiodigalactoside, (a potent inhibitor of the lectin's activity) to media containing freshly prepared cell suspensions in stationary culture results in decreased intercellular and cell-substratum adhesion (Cook et al., 1979; Milos et al., 1980; Milos and Zalik, 1981). This suggested that the lectin may be deposited extracellularly and may have a role in cell-substratum and cell-cell adhesion. The blastoderm lectin has been localized immunohistochemically to the interior of cells from the endoderm of the area opaca. It was also localized to extracellular sites of cell detachment from the substratum (Zalik et al., 1982).

In early studies on the reaggregation of cells dissociated from the unincubated blastoderm (Zalik and Sanders, 1974), it was found that hypoblast cells sorted out from epiblast cells, to the outer region of the aggregate. These cells were determined to be hypoblast because of their morphological similarity to yolk sac endoderm. This was later confirmed by Eyal-Giladi et al. (1975). In a later study using gastrula stage embryos, similar results were obtained in that the cells underwent

cell sorting and differentiation into several recognizable cell types including yolk sac endoderm (Sanders and Zalik, 1976). Milos et al. (1979) developed an approach to studying selective cell adhesion in early embryonic cells that involved the isolation of a pure population of single cells. Cells from the extraembryonic endoderm were obtained from gastrula stage embryos and their aggregative behavior and subsequent morphogenesis were studied. These cells were found to aggregate readily when cultured in rotating flasks. With further development, they cavitate and differentiate into fluid filled vesicles surrounded by a single layer of cells that resembles the yolk sac endoderm (Milos et al., 1979, 1984). This ability of endoderm cells appears to be intrinsic because other cell types are not present in the aggregate. These workers believe that such aggregates may be a relevant in vitro model system for the study of cell-cell interactions during yolk sac formation in vivo.

4. PURPOSE OF STUDY

As mentioned previously, the developing yolk sac of the chicken embryo expands to surround the yolk mass by processes of proliferation and migration. Such cell movements probably involve cell-cell interactions between cells of the extraembryonic endoderm that are mediated by specific cell surface macromolecules. The possibility that endogenous lectins may play some role in these migratory interactions exists.

As a first approach to the problem it was necessary to determine if any endogenous lectins are present in the extraembryonic area opaca as it develops. As mentioned previously, it has been determined that endogenous β-D-galactoside-binding lectins and cell surface receptors bearing terminal galactosyl groups are present in the endodermal cells of the area opaca of gastrulating chicken embryos (Cook et al., 1979; Phillips and Zalik, 1982; Zalik et al., 1983). These cells are the precursors of the yolk sac epithelium. It was of interest to determine if similar lectin activities are still present at later developmental stages, since this tissue is in the process of epiboly, with cells becoming rearranged from a multilayered cell group and differentiating to form a single cell layer around the yolk.

This study was undertaken to investigate the changes in these lectin levels during development of the yolk sac and to determine if such changes were under developmental regulation. Lectin activity was obtained from soluble extracts of the area vitellina and area vasculosa of the developing yolk sac of chicken embryos, at successive stages of development up to 7 days of incubation. Levels of activity were compared in the area vitellina and area vasculosa, at the different stages of development. Further work involved the isolation and characterization of lectins from the area vitellina and area vasculosa tissues of 2-day-old yolk sac. Possible relationships between these lectins and the two galactoside-binding lectins isolated from primitive streak stage tissues were evaluated. The results indicate that yolk sac lectins, which appear to be identical in the two areas, are similar to

the blastoderm lectins in saccharide specificity, heterogeneity on gel filtration chromatography, and subunit molecular weight in sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The presence of these lectins at this stage of development and at later stages, with continued differentiation of the yolk sac endodermal layer suggests that they could have a role to play in the differentiation of this extraembryonic tissue.

MATERIALS AND METHODS

1. Preparation of Embryos

Fertilized eggs from White Leghorn chickens were obtained from the University of Alberta poultry farm. The eggs were incubated at 39°C for 48-50 hours or for 3, 4, 5, 7 and 16 days, to yield embryos at the various developmental stages used in this study. These incubation times yielded stages 13, 14 and 15 (48-50 hours), stage 18 (3 days), stage 22 (4 days), stage 26 (5 days), stage 30 (7 days) and stage 42 (16 days) embryos, staged according to Hamburger and Hamilton (1951).

Embryos, with their accompanying area opacae or yolk sacs were cut off the yolk as close to the outer margin of the area vitellina as possible and placed at room temperature in Tyrode's saline solution, pH 7.8 (0.14 M NaCl, 0.003 M KCl, 0.002 M $CaCl_2$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.4 mM $NaH_2PO_4 \cdot 2H_2O$, 0.012 M $NaHCO_3$, 0.006 M glucose.) The vitelline membranes were removed, and embryos with their area opacae or yolk sac membranes were washed extensively to remove adherent yolk granules. Embryos at the correct stage of development were selected by examination under a dissecting microscope.

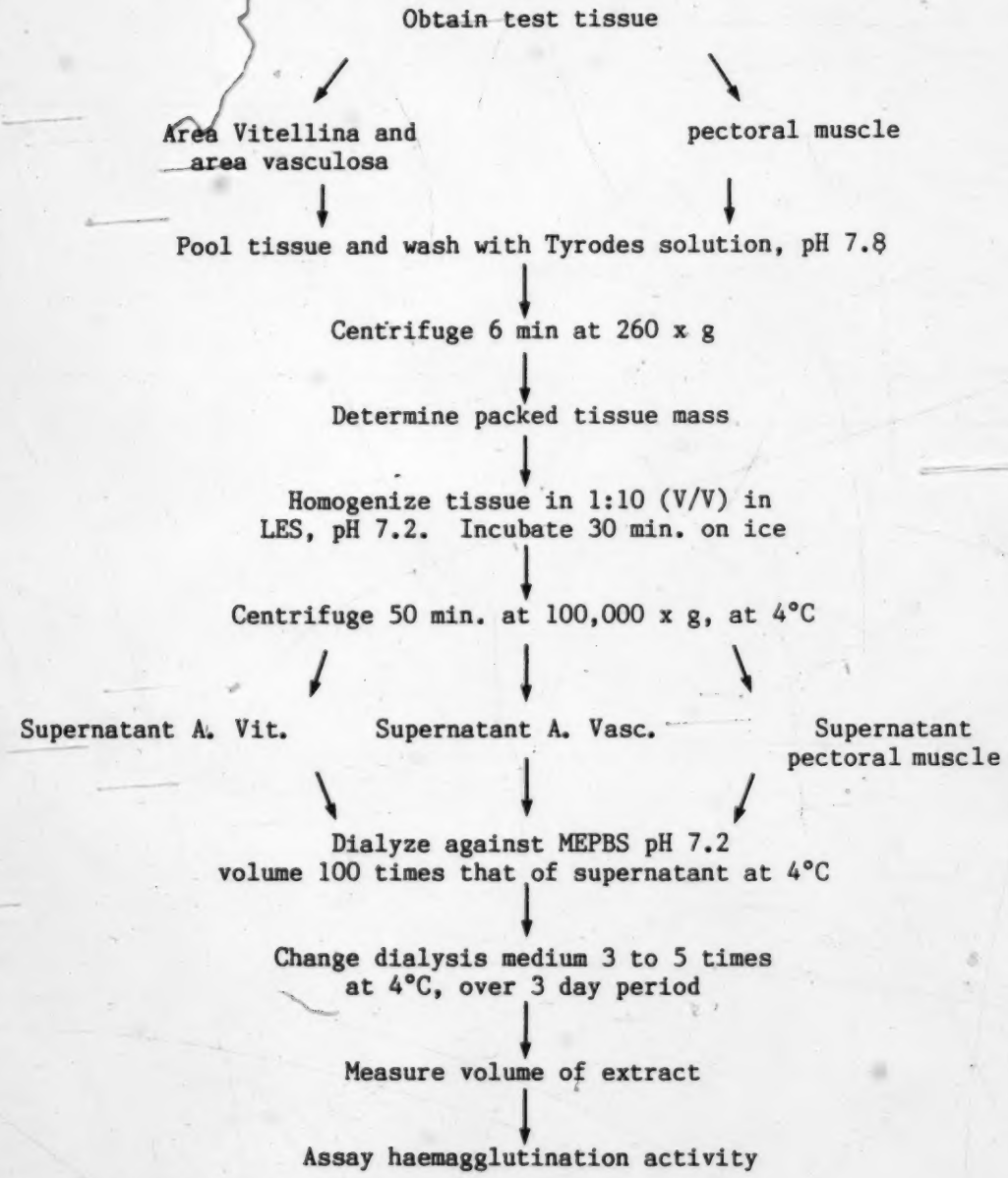
2. Preparation of Tissue Extracts

Embryos were dissected out with fine irridectomy scissors and discarded. The two regions of the area opaca, the outer area vitellina (a. vit.) and the medial area vasculosa (a. vasc.) were separated by

cutting along the outer border of the sinus terminalis, a large distinct blood vessel that encircles the outer margin of the area vasculosa (Romanoff, 1960). For embryos at stages 13-15, tissue from approximately 200 area vitellinae and area vasculosae were pooled and transferred to a 15 ml graduated centrifuge tube. For more advanced stages, fewer embryos were used; usually a. vit. and a. vasc. tissue from approximately nine embryos (stage 18 and 22) and from approximately five embryos (stages 26 and 30). For 16 day-old embryos, pectoral muscles were excised from four to five embryos. Pooled tissues were washed once and centrifuged for 6 minutes at 260 x g in a clinical centrifuge, to determine the packed tissue mass.

The method used for lectin extraction is by Zalik et al. (1983) as modified from Nowak et al. (1976) and is illustrated in a flow chart in Figure 2. The washed tissues were homogenized in cold lectin extraction solution (LES) pH 7.2 (0.3 M lactose; 0.002 M ethylenedinitrilotetraacetic acid (EDTA); 0.02% sodium azide (NaN_3); 0.25 mM phenylmethylsulphonyl fluoride (PMSF); in 0.005 M phosphate buffer, 0.15 M NaCl, 0.004 M 2-mercaptoethanol (2ME). Tissues were disrupted with a Potter-Elvehjem tissue homogenizer (Bellco) and for pectoral muscle tissues, a Sorvall-Omni mixer was used. The ratio of extraction solution to packed tissue mass was 10:1 (V/V). The homogenates were left on ice for 30 minutes and then centrifuged at 100,000 x g for 50 minutes in a swinging bucket rotor in a preparative ultracentrifuge (Beckman model L2-65B) at 4°C. Supernatants were either stored undialyzed at -20°C or used immediately. For dialysis, extracts were placed in membrane dialysis tubing (Spectrapor No. 1; MW cut off point: 8,000) which had been

Figure 2. Procedure for the Extraction of Lectin Activity


Obtain test tissue
Area Vitellina and area vasculosa
pectoral muscle
Pool tissue and wash with Tyrodes solution, pH 7.8
Centrifuge 6 min at 260 x g
Determine packed tissue mass
Homogenize tissue in 1:10 (V/V) in LES, pH 7.2. Incubate 30 min. on ice
Centrifuge 50 min. at 100,000 x g, at 4°C
Supernatant A. Vit.
Supernatant A. Vasc.
Supernatant pectoral muscle
Dialyze against MEPBS pH 7.2 volume 100 times that of supernatant at 4°C
Change dialysis medium 3 to 5 times at 4°C, over 3 day period
Measure volume of extract
Assay haemagglutination activity

previously soaked in distilled water. Extracts were dialyzed exhaustively against mercaptoethanol-phosphate buffered saline (MEPBS) pH 7.2 (consisting of 0.005 M phosphate buffer, 0.15 M NaCl, 0.004 M 2ME). Dialysis was performed for two to three days against three to five changes of medium in the cold room at 4°C. The resulting lactose-free crude extracts were used in further experiments.

In initial studies the suitability of various extraction solutions for the extraction and preservation of lectin activity was determined. For this purpose lectin activity was studied in the presence and absence of 2ME (0.004 M), lactose (0.3 M), $CaCl_2$ (0.002 M) and $MgCl_2$ (0.002 M). These experiments were performed in tissues of the a. vit. and a. vasc. from embryos at stages 13–15.

3. Assay of Lectin Activity

The procedure for the preparation of test erythrocytes, used in this study is illustrated in Figure 3 (Zalik et al., 1983). For the semiquantitative detection of lectin activity in extracts, an haemagglutination assay using rabbit erythrocytes was used (Nowak et al., 1976). The assay uses a standard serial two-fold dilution of extract to be tested, in agglutination saline (0.15 M NaCl in 0.005 M phosphate buffer, pH 7.2). This was performed in microtiter 'V' plates (Cooke Engineering). For each extract to be tested, titrations were carried out in duplicate. Twenty-five microliters of agglutination saline was pipetted into a serial row of wells, then 25 μl of tissue extract was added to the first well and serially diluted by pipetting

Figure 3. Procedure for the Preparation of Test Erythrocytes for the Haemagglutination Assay

Collect 10 ml rabbit blood in 20 ml Alsever's solution (0.114 M dextrose, 0.027 M Sodium citrate 0.026 M citric acid, 0.072 M NaCl)

↓

Centrifuge blood 10 min. at 900 x g in a clinical centrifuge

↓

Wash cells 4 times with 5 volumes of 0.15 M NaCl in 0.005 M phosphate buffer, pH 7.2

↓

Estimate volume of packed cells after fourth wash

↓

Resuspend in 1:25 (V/V) freshly prepared trypsin solution (1 mg/ml trypsin in 0.05 M NaCl, 0.1 M phosphate buffer, pH 7.2) (Trypsin Type III from bovine pancreas, Sigma Chemical Co.)

↓

Incubate in gyrotory water bath, 60 min. at 37°C at approximately 145 rpm

↓

Centrifuge blood 10 minutes at 900 x g

↓

Wash cells 4 times with 5 volumes of 0.075 M NaCl in 0.075 M phosphate buffer, pH 7.2, containing 0.02% NaN_3 and 0.25 mM PMSF

↓

Estimate final volume of packed cells after fourth wash

↓

Resuspend cells to 10% (V/V) suspension with 0.075 M NaCl in 0.075 M phosphate buffer, pH 7.2, containing 0.02% NaN_3 and 0.25 mM PMSF

↓

For haemagglutination assay, dilute erythrocyte suspension to 4% suspension in 0.15 M NaCl in 0.005 M phosphate buffer, pH 7.2

↓

Erythrocytes prepared by this method are usable for up to 10 days. The reproducibility of the agglutination results in different batches of erythrocytes was routinely tested with peanut agglutinin (Miles-Yeda)

and mixing of sample through the row of wells containing the agglutination saline. This was followed by the addition of 50 μl of 0.5% bovine serum albumin in saline (BSA) to each well and finally 25 μl of a 4% rabbit erythrocyte suspension was added. All solutions were dispensed with a Titertek multichannel pipette. The plates were agitated vigorously to mix the cells and reagents, and incubated at room temperature for 60 minutes. Patterns of agglutination were then determined. Agglutinated erythrocytes formed a diffuse mat on the bottom of the well, whereas non-agglutinated cells generally formed a compact dot (Figure 4).

Haemagglutination activity is expressed as titer, which is the reciprocal of the maximal dilution of extract that gives visible agglutination of test erythrocytes. Controls consisted of erythrocytes mixed with 25 μl agglutination saline instead of tissue extracts. They consistently showed a compact dot on the bottom of the well (Figure 4).

4. Saccharide Inhibition Assay

To determine the carbohydrate specificity of lectin extracts, different saccharides were tested for their ability to inhibit haemagglutination. The following sugars were obtained from the Sigma chemical company: thiodigalactoside (TDG), lactose, galactose, phenyl-β-D-galactopyranoside, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-mannopyranoside, α-D-melibiose, N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc), D-

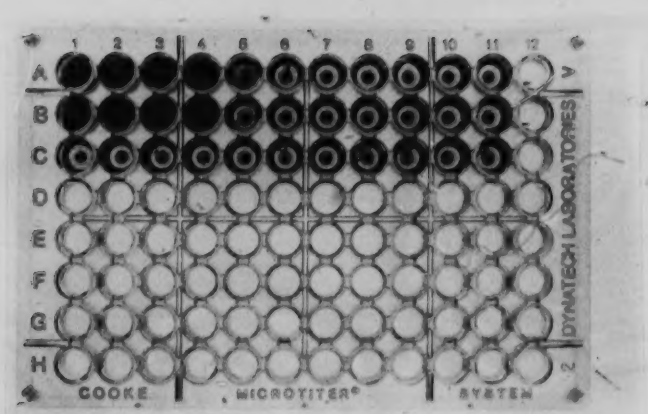


Figure 4

Titration plate illustrating the appearance of agglutinated and unagglutinated trypsinized rabbit erythrocytes in a haemagglutination assay. The lectin titer in row A and B is 16. Positive agglutination can be observed as a mat of cells in bottom of wells in rows A and B. Observe the compact dots of unagglutinated cells in control row C, which contains saline only.

fucose and L-fucose. (2R)-glycerol-O-β-D-galactopyranoside (RGG) and isopropyl-β-D-thiogalactopyranoside were obtained from Calbiochem.

The sugar inhibition assay used was basically the same as the haemagglutination assay with the exception that a sugar hapten, dissolved in agglutination saline to an appropriate concentration, was used in place of agglutination saline alone. Extracts of crude or purified lectin from the area vitellina and the area vasculosa, as well as from 16-day pectoral muscle were serially diluted in the sugar solution. The plates were mixed and incubated for 30 minutes at room temperature before the addition of 0.5% BSA and 4% rabbit erythrocytes. Controls using agglutination saline without sugar were treated similarly. All titrations were carried out in duplicate.

The ability of saccharides at various concentrations to inhibit at least 4 haemagglutinating units (HU) of lectin was tested. Extracts were diluted to a titer of either 8 or 4 HU and the concentration of saccharide that either reduced the titer from 8 to 4 HU or abolished 4 HU completely was assessed. The saccharide concentrations tested were as follows: TDG (0.025 to 0.4 mM); lactose (0.125 to 1 mM); RGG (0.25 to 12.5 mM); isopropyl-β-D-thiogalactopyranoside (1.25 to 50 mM); phenyl-β-D-galactopyranoside and methyl-α-D-galactopyranoside (2.5 to 50 mM); methyl-β-D-galactopyranoside (2.5 to 100 mM); α-D-melibiose (5 to 100 mM); galactose (10 to 75 mM); and methyl-α-D-mannopyranoside, GalNAc, GlcNAc, D-fucose and L-fucose (10 to 100 mM).

5. Protein Determination

Protein concentrations of crude and purified lectin extracts from a. vit. and a. vasc. tissue as well as 16-day pectoral muscle were determined by the method of Bradford (1976) as described in the Biorad laboratory protein determination kit. A series of standards consisting of bovine gamma globulin at concentrations varying gradually from 0.2 to 1.4 mg/ml (for macroassay) and 1 to 25 μg/ml (for microassay) were prepared according to the instruction manual. Standards and samples in a volume of either 0.1 ml or 0.8 ml sample buffer, respectively, were placed in test tubes to which 5 ml of diluted dye reagent or 0.2 ml concentrated dye reagent, respectively, were added. A blank containing sample buffer was also prepared. The solutions were mixed by gentle vortexing and absorbance at 595 nM was read in a Beckman DU-8 spectrophotometer. A standard curve of OD_{595} against the concentration of standards was used to determine the protein concentrations of the unknown samples.

The specific activity of the crude tissue extracts or purified lectin was calculated as HU/mg protein by dividing the number of haemagglutinating units by the concentration of protein in an extract.

6. Gel Filtration Chromatography

Lectin activity was further characterized by gel filtration chromatography on Sephacryl S-200 superfine (Pharmacia). This gel matrix has a fractionation range for proteins of molecular weight $5 \times 10^3 - 2.5 \times 10^5$. A column (2.6 cm diameter x 35 cm length) was

prepared according to the manufacturers instructions, and packed at a flow rate of 99 ml/h. The column was connected via a peristaltic pump (LKB microperpex) to a UV monitor with recorder (LKB 2138 Uvicord S and LKB 2210 potentiometric recorder) and a fraction collector (LKB 2111 Multirac). The flow rate used for application of the protein standards was 20 ml/h and that of the lectin sample was 20–25 ml/h and fractions of a 4 ml volume were collected.

The protein standards used to calibrate the column were from Pharmacia Fine Chemical's low molecular weight gel filtration calibration kit, with the exception of cytochrome C, which was obtained from Sigma. Ferritin (MW 440,000) was used to determine the void volume of the bed, which is equal to the elution volume of excluded material, and because ferritin is a colored protein, it was useful in determining the uniformity of the bed. The protein standards were as follows: cytochrome C (MW 12,400); ribonuclease A (MW 13,700); ovalbumin (MW 43,000) and bovine serum albumin (MW 67,000).

The elution volume of each protein was determined according to the manufacturers instructions (Pharmacia). The value for the elution volume (Ve) for each standard and the values for total bed volume (Vt) and void volume (Vo) of the column were used to calculate the parameter, Kav, a partition coefficient, using the formula Kav = Ve - Vo/Vt - Vo (Pharmacia instruction manual). A plot of Kav versus Log MW of the protein standards gave a standard curve that was used to determine the molecular weights of the unknown samples.

Crude extracts of a. vit. and a. vasc. tissue (2 ml) containing 0.3 M lactose were applied to the column and eluted with MEPBS containing 0.3 M lactose. Three separate preparations of these extracts were characterized. All of the collected fractions were tested for haemagglutination activity, and elution volumes were determined. As with the standards, the Ve values were used to determine the Kav values.

7. Lectin Purification by Affinity Chromatography

Lectin activity was purified by affinity chromatography on a column of Sepharose 4B (Pharmacia) to which para-aminophenyl-β-D-lactoside was linked, by the method of Nowak et al. (1977). The method for derivatization of the column involved activation of Sepharose 4B with cyanogen bromide, at alkaline pH and the linking of the coupling agent hexanediamine, an aliphatic diamine compound. The free amino groups supplied by this compound were succinylated by treating the amino-alkyl Sepharose with succinic anhydride at pH 6.0 in aqueous media, which increased the length of the spacer arm. The succinyl-aminoalkyl Sepharose at a 50% suspension was then treated with the ligand para-aminophenyl-β-D-lactoside (APL), (Vega-Fox Biochemicals) in the presence of 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide at acid pH. Finally the resin of APL-coupled Sepharose 4B was incubated with 1M ethanolamine at 4°C and alkaline pH, to block residual active groups remaining exposed on the gel after coupling. At each step, color tests were carried out to confirm completion of each chemical reaction. Excess uncoupled ligand was removed by washing five times, alternately with bicarbonate buffer (0.1 M, pH 9.1) and acetate buffer (0.1 M, pH

4.0), each containing 1M NaCl. The APL-coupled Sepharose was resuspended in MEPBS, pH 7.2 containing NaN_3, poured into a Pharmacia column (1.6 cm diameter x 14 cm length) and packed using MEPBS at a flow rate of 25 ml/h.

For purification, lactose free lectin extracts from tissue of the area vitellina (10-20 ml) or of the area vasculosa (7-14 ml), or 16-day pectoral muscle (16 ml) were applied to the column at a flow rate of 25 ml/h, in the cold (4°C). The column was washed successively with approximately 100 ml MEPBS, 100 ml of 0.3 M sucrose in MEPBS, 100 ml MEPBS, 100 ml of 0.3 M lactose in MEPBS and then MEPBS, until the baseline was reached. Fractions (3.6 ml) were checked for haemagglutination activity either immediately (those in MEPBS) or after dialysis against MEPBS (those in sucrose and lactose). Fractions containing lectin activity were dialyzed against distilled water containing 0.004 M 2ME, frozen at -70°C and lyophilized. Samples were stored at -20°C until they were used in sodium dodecyl sulphate-polyacrylamide gel electrophoresis or isoelectric focusing. Purified lectin samples (before lyophilization) were also used in the saccharide inhibition assays.

8. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The subunit molecular weight of the lectin(s) purified from extracts of a. vit. and a. vasc. tissue and 16-day pectoral muscle was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Acrylamide and N, N, N', N'-tetramethylethylenediamine

(TEMED) were obtained from Eastman Kodak; N, N'-methylenebisacrylamide (Bis), urea, sodium dodecyl sulphate (SDS) and Coomassie Brilliant Blue R-250 (CBB) were obtained from Biorad. Other chemicals used include: Tris (hydroxymethyl)aminomethane, glycine and ammonium persulphate, analytical grade, obtained from Fisher and 2-mercaptoethanol was from Sigma. Glacial acetic acid, methanol and isopropyl alcohol were reagent grade chemicals.

Horizontal thin-layer slab gels of 11% gel concentration were prepared by the method of Zalik et al. (1983) as adapted from Weber and Osborn (1969). Starting with an acrylamide-bisacrylamide stock solution (60% acrylamide, w/v; 1.6% Bis, w/v in distilled water), 60 ml of an 11% acrylamide solution was prepared by first mixing 10.7 ml acrylamide stock with 19.3 ml distilled water. This solution was degassed under vacuum for 5 minutes. Then 0.2% ammonium persulphate in 0.4% aqueous SDS solution at a volume of 15 ml and 15 ml TEMED solution (0.13% TEMED, v/v; 18.2% Tris, w/v, in distilled water, pH 8.3) were mixed carefully with the acrylamide solution and immediately poured into a gel mold. This mold was made up of a plastic slot former and a thin glass plate (125 x 260 x 1 mm) separated by a 2 mm thick rubber gasket (all from LKB). Metal clamps were used to hold the mold together with a thick glass plate (125 x 260 x 3 mm) supporting the thin plate. Gels were allowed to polymerize overnight at room temperature.

After polymerization, the gel on the thin glass plate support was placed on the cooling plate of an LKB multiphor unit (model 2117) that had been cooled to 5°C by a Polytemp cooling system (Polyscience). The reservoir buffer used was an aqueous Tris-Glycine buffer (0.3% Tris,

w/v; 1.4% glycine, w/v), containing 0.1% SDS (w/v), pH 8.3 in which the electrodes and electrode wicks (LKB) were immersed. The wicks were applied to either side of the gel length with an overlap of about 15 mm. With the anti-condensation lid in place gels were pre-electrophoresed for 2 hours to remove impurities.

Lyophilized protein samples were solubilized in sample buffer. Two sample buffers were used in four initial experiments run concurrently and in subsequent experiments only the reducing buffer was used. The reducing buffer contained 2% SDS, 0.05 M Tris, 5% 2ME and 8M Urea in distilled water, pH 8.3 and non-reducing buffer contained 2% SDS and 0.05 M Tris in distilled water, pH 8.3. In addition 0.3 M lactose was added to each sample buffer prior to solubilization of samples. The protein samples were incubated at 60°C for 60 minutes. Protein concentration was normally between 0.2 and 0.9 μg per μl of lectin protein from both area vitellina and area vasculosa and 0.8 μg per μl of lectin protein from 16-day pectoral muscle.

The following were the standards run in each gel: a low molecular weight mixture of proteins from Biorad containing lysozyme (MW 14,400), soybean trypsin inhibitor (MW 21,500), carbonic anhydrase (MW 31,000), ovalbumin (MW 45,000), BSA (MW 67,000) and phosphorylase B (MW 92,500) at a concentration of 0.01 μg per μl for each standard. In addition, individual standards obtained from Pharmacia, at similar concentrations including BSA, ovalbumin, chymotrypsinogen A (MW 25,000), ribonuclease A (MW 13,700) and cytochrome C (MW 12,400) were used. All standards were

solubilized in reducing buffer, boiled for 5 minutes and a drop of 0.5% bromophenol blue dye (Fisher) was added to each.

Both samples and standards at a volume of 15 μl were applied to slots in the gel. Gels were electrophoresed for 4 to 5 hours. A voltage of 50 v and a pulsed current of 50 pulses per second (pps), (Ortec 4,100 pulsed constant power supply) were applied until the samples were seen to move out of the slots. Then the voltage and pulsed current were gradually increased at one-half hour intervals until a value of 300 v and 300 pps was attained.

Following electrophoresis gels were placed in an aqueous 0.04% Coomassie blue stain (0.04% CBB, w/v, in 10% acetic acid, v/v, and 25% isopropyl alcohol, v/v), and gently shaken at room temperature overnight. Background staining was removed by washing the gels in a destaining solution (containing 10% acetic acid, v/v, and 10% isopropyl alcohol, v/v, in distilled water) with several changes (method adapted from Fairbanks et al., 1971).

Gel measurements were made both before and after staining. The distances migrated by the bands produced by the standards, as well as by the sample proteins were measured. Mobility (M_R) values for standards were calculated as follows:

$$M_R = \frac{\text{distance travelled by protein}}{\text{distance travelled by dye}} \times \frac{\text{length gel before staining}}{\text{length gel after staining}}$$

This calculation of the M_R corrects for change in gel length due to swelling. M_R values were plotted against the known Log MW values of the protein standards to give a standard curve. The standard curve obtained by linear regression analysis was used to determine the molecular

weights of the unknowns using M_R values. Gels were usually photographed with a Nikon FE 35 mm camera using Kodak panatomic-X film and an orange filter.

In some experiments, to further visualize protein bands a silver staining method by Wray et al. (1981) was used after Coomassie blue staining. Gels were fixed in an aqueous solution containing 50% methanol, and 10% acetic acid (v/v/v) overnight and then incubated for 1 hour in an aqueous solution of 5% methanol and 7% acetic acid (v/v/v). This was followed by incubation in an aqueous solution of 5% glutaraldehyde (v/v) for 1 hour and exhaustive washing in distilled water for 2 hours. This initial treatment of the gels, suggested by Oakley et al. (1980) minimizes background staining and enhances protein band staining. Gels were then stained in an ammoniacal silver solution prepared as follows: 8 ml of silver nitrate solution (1.6 g $AgNO_3$ in 8 ml distilled water) was added, dropwise, to 42 ml of 0.36% sodium hydroxide and 2.8 ml concentrated ammonium hydroxide, and made up to 200 ml with distilled water. After 15 minutes in staining solution, gels were washed in distilled water for 5 minutes and citric acid developer (2.5 ml of an aqueous 1% citric acid solution, w/v, and 0.05% formaldehyde, v/v, in distilled water) was added and the gels were allowed to remain in this solution for 4 to 5 minutes at which time bands had usually become visible. In some cases high background staining was removed by washing briefly (5 to 10 minutes) in Kodak rapid fix (film strength) and then incubating for 1 hour in Kodak hypoclear (1:4, v/v) to remove rapid fix. Gels were washed briefly and placed in

50% methanol (v/v) for 30 minutes to stabilize band staining. Molecular weights were determined as in gels stained with Coomassie blue.

9. Analytical Isoelectric Focusing in Thin-Layer Polyacrylamide Gels

Electrofocusing in thin-layer polyacrylamide gels by the method of Cook (1976) was used to determine the isoelectric points of lectin samples. Acrylamide, Bis, and TEMED used were the same as for SDS-polyacrylamide gel electrophoresis. Riboflavin was purchased from Eastman Kodak and Ampholine, pH range 3.5 to 10, was obtained from LKB.

Horizontal thin-layer slab gels of 6.8% gel concentration were prepared as follows: 14.7 ml of acrylamide-bisacrylamide stock solution (28% acrylamide, w/v; 0.7% Bis, w/v, in distilled water), 1.8 ml of ampholine and 41.4 ml distilled water were mixed and degassed under vacuum for 10 minutes. A volume of 4.2 ml of catalyst (0.007% riboflavin, w/v; 0.7% TEMED, v/v, in distilled water) was carefully added to the acrylamide solution, mixed and poured into a gel mold (LKB), and left overnight to polymerize under fluorescent light. This results in a 2 mm thick gel slab. Electrode strips (LKB) were moistened with either 1M potassium hydroxide for the cathode or 1M phosphoric acid for the anode. Standards were obtained from Pharmacia's isoelectric focusing calibration kit and resuspended in 1% glycine (w/v in distilled water). They included the following: amyloglucosidase (pI 3.5), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin, acidic (pI 6.85) and basic (pI 7.35), lentil lectin trimer (pI 8.15, 8.45 and 8.65) and trypsinogen (pI 9.3). Samples (30-

80 μg) and standards (20-50 μg) were loaded on 10 mm x 5 mm rectangular pieces of cambrelle (Industrial Chemical Company) at volumes ranging from 50 to 100 μl and applied to the gel approximately 3 cm from the cathode wick. With the electrofocusing lid in place, focusing was performed over the length of the gel (25 cm) for 4 to 6 hours at a constant temperature of 10°C. Voltage was increased from 600 to 1200 v during the first hour, while the current decreased from approximately 20 mA to about 5 mA at the end of focusing (LKB 3371E DC power supply). Gels were fixed in 12.5% trichloracetic acid (w/v in distilled water) for 18 hours and then soaked in distilled water. Several washes with distilled water preceded overnight staining of the gels in a 0.04% CBB stain (0.04% CBB, in ethanol-acetic acid-distilled water, 5:2:5, v/v). Gels were washed several times in destaining solution consisting of ethanol-glycerol-acetic acid-distilled water (5:5:2:13; v/v).

Photographs were taken, and band distances for standards and samples were measured. The pI values of the proteins used as standards were plotted against the distance migrated from the cathode by the bands produced by these proteins. A standard curve of pH versus distance migrated from the cathode was used to determine pI values for the unknowns.

The silver staining method used in SDS-polyacrylamide gel electrophoresis was also used to further visualize bands and pI values were calculated in the same way.

10. Histology

Samples of area opaca tissue containing representative regions of the a. vit. and a. vasc. were dissected out from 2d, 3d, 4d, 5d and 7d embryos, washed and positioned on the concave surface of a watch glass for fixing. Tissues were fixed in Bouins fixative (an aqueous solution containing picric acid-formalin-acetic acid, 15:5:1, v/v) overnight, dehydrated in a series of alcohols and embedded in paraplast. The procedure used is illustrated in a flow diagram in Figure 5. Paraffin blocks were mounted on specimen holders and 5-7 μm sections were cut using a rotary microtome (American Optical, No. 820). Sections were deparaffinized and stained with Haematoxylin and Eosin, using the procedure outlined in a flow diagram in Figure 6.

Figure 5. Procedure for Embedding Area Opaca Tissue

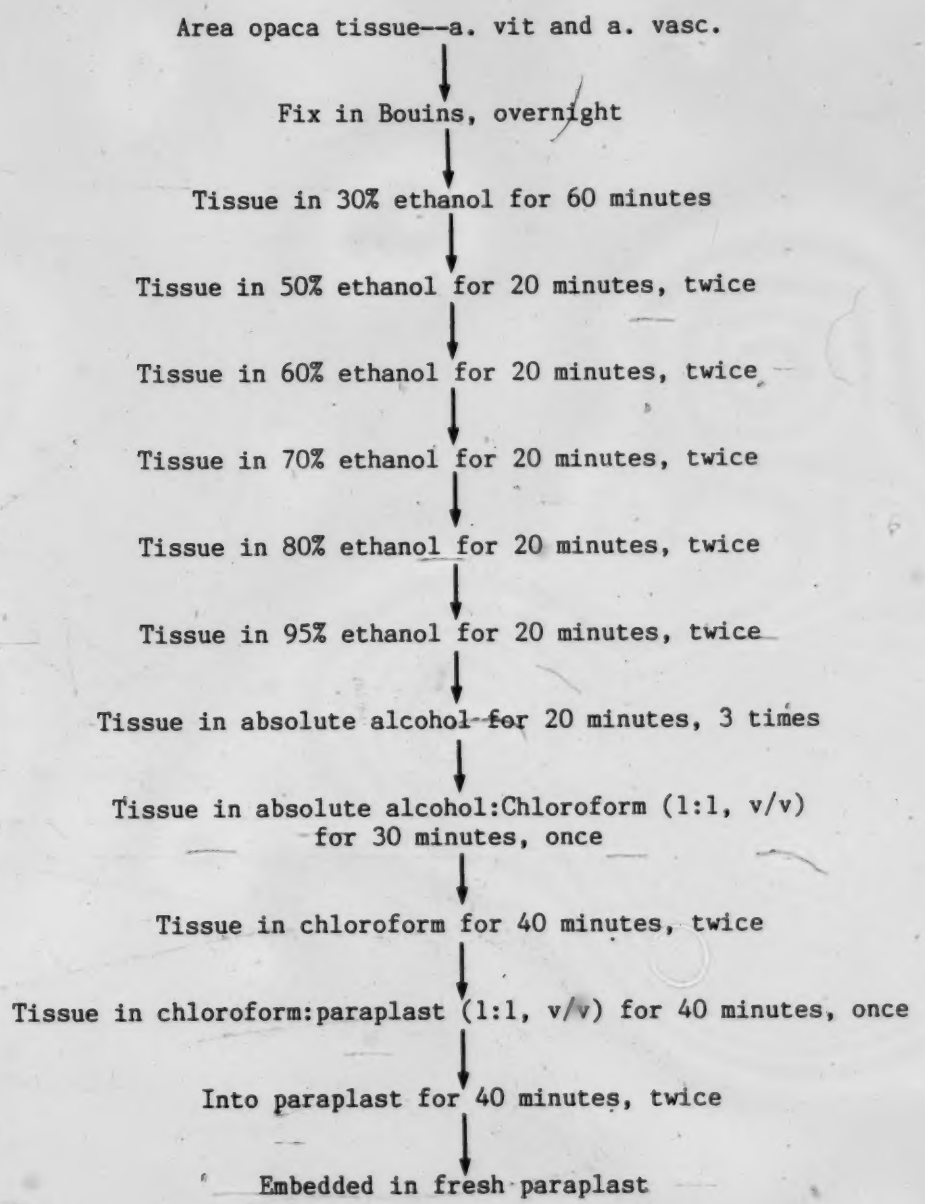

Area opaca tissue--a. vit and a. vasc.
Fix in Bouins, overnight
Tissue in 30% ethanol for 60 minutes
Tissue in 50% ethanol for 20 minutes, twice
Tissue in 60% ethanol for 20 minutes, twice
Tissue in 70% ethanol for 20 minutes, twice
Tissue in 80% ethanol for 20 minutes, twice
Tissue in 95% ethanol for 20 minutes, twice
Tissue in absolute alcohol for 20 minutes, 3 times
Tissue in absolute alcohol:Chloroform (1:1, v/v) for 30 minutes, once
Tissue in chloroform for 40 minutes, twice
Tissue in chloroform:paraplast (1:1, v/v) for 40 minutes, once
Into paraplast for 40 minutes, twice
Embedded in fresh paraplast

Figure 6. Procedure for Haematoxylin and Eosin Staining of Embedded Area Opaca Tissue

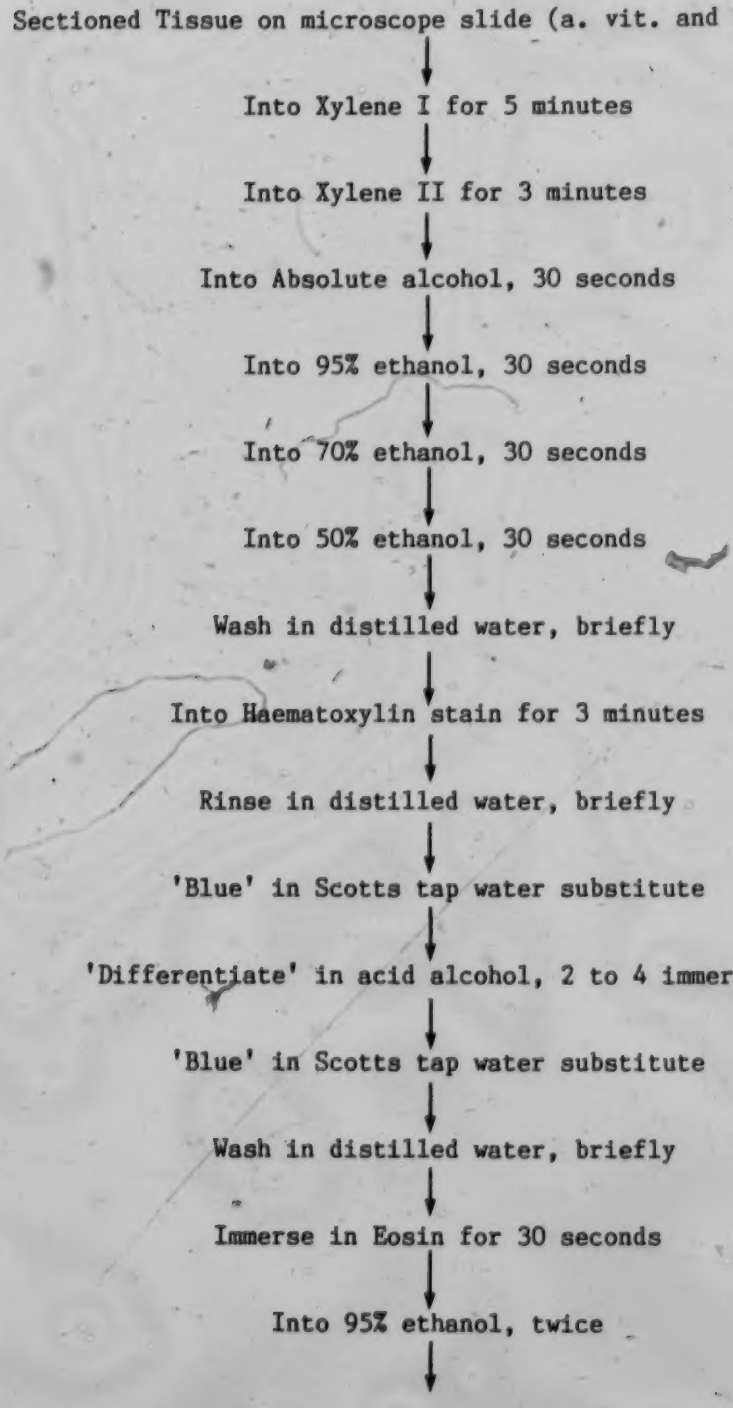
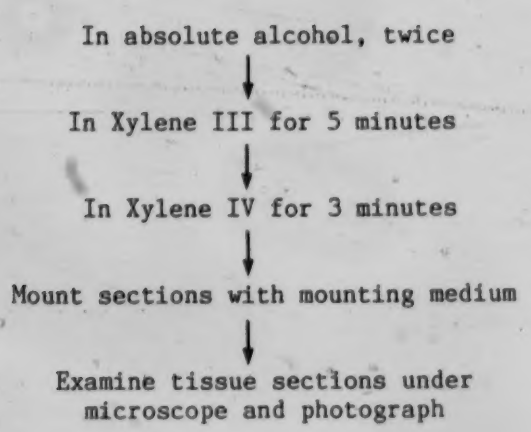

Sectioned Tissue on microscope slide (a. vit. and a. vasc.)
Into Xylene I for 5 minutes
Into Xylene II for 3 minutes
Into Absolute alcohol, 30 seconds
Into 95% ethanol, 30 seconds
Into 70% ethanol, 30 seconds
Into 50% ethanol, 30 seconds
Wash in distilled water, briefly
Into Haematoxylin stain for 3 minutes
Rinse in distilled water, briefly
'Blue' in Scotts tap water substitute
'Differentiate' in acid alcohol, 2 to 4 immersions
'Blue' in Scotts tap water substitute
Wash in distilled water, briefly
Immerse in Eosin for 30 seconds
Into 95% ethanol, twice

Figure 6, continued


In absolute alcohol, twice
In Xylene III for 5 minutes
In Xylene IV for 3 minutes
Mount sections with mounting medium
Examine tissue sections under microscope and photograph

RESULTS

1. Histological Examination of Area Vitellina and Area Vasculosa Tissue from 2 Day, 3 Day , 4 Day, 5 Day and 7 Day Yolk Sac Membranes

Histological sections of the a. vit. and a. vasc. were prepared from 2day, 3day, 4day, 5day and 7day yolk sacs. Representative sections are shown in Figures 7-11. These light micrographs show the structure of these tissues at subsequent stages of development.

During the spreading of the yolk sac over the surface of the yolk mass, it consists of a medial vascularized region, the area vasculosa and a peripheral, non-vascular region, the area vitellina. This latter tissue consists of ectoderm and endoderm and shortly after being invaded by mesoderm is transformed into the area vasculosa. Sections taken from within the area vasculosa (Figures 7A, 8A, 9A, 10A, 11A) show the three cell layers; ectoderm, mesoderm and endoderm. The ectoderm in this region is usually a monolayer of squamous cells which may overlap to some extent. At progressively later stages of development, the organization of this cell layer remains much the same. Occasionally the ectodermal cells are observed to be thickened and appear to have assumed a bilayered arrangement. In some instances the ectoderm has separated from the endoderm leaving a space between these two cell layers. The mesoderm in the region of the area vasculosa is composed of two distinct components, the somatic mesoderm and the splanchnic mesoderm. The

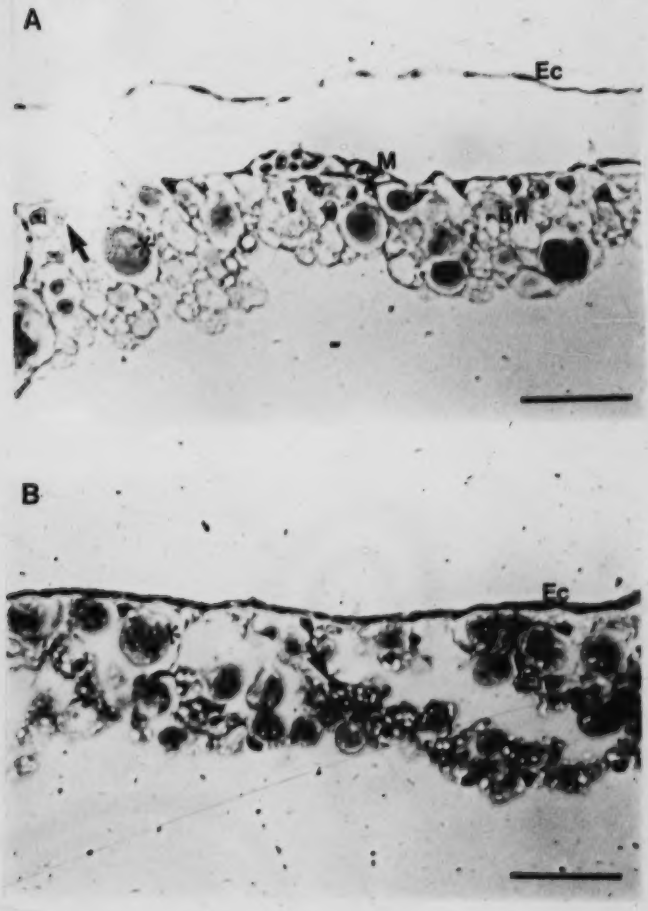


Figure 7.

Light micrographs of representative sections through the area vasculosa (A) and the area vitellina (B) from the yolk sac of a 2-day chick embryo. The cell layers present in both of these areas are the ectoderm (Ec) and the endoderm (En). A third cell layer, the mesoderm (M) containing blood vessels can be observed in the area vasculosa only. Observe the endodermal nuclei (arrows) in both A and B. Large yolk granules (*) can also be seen in the endoderm. The bar is 50 μm.

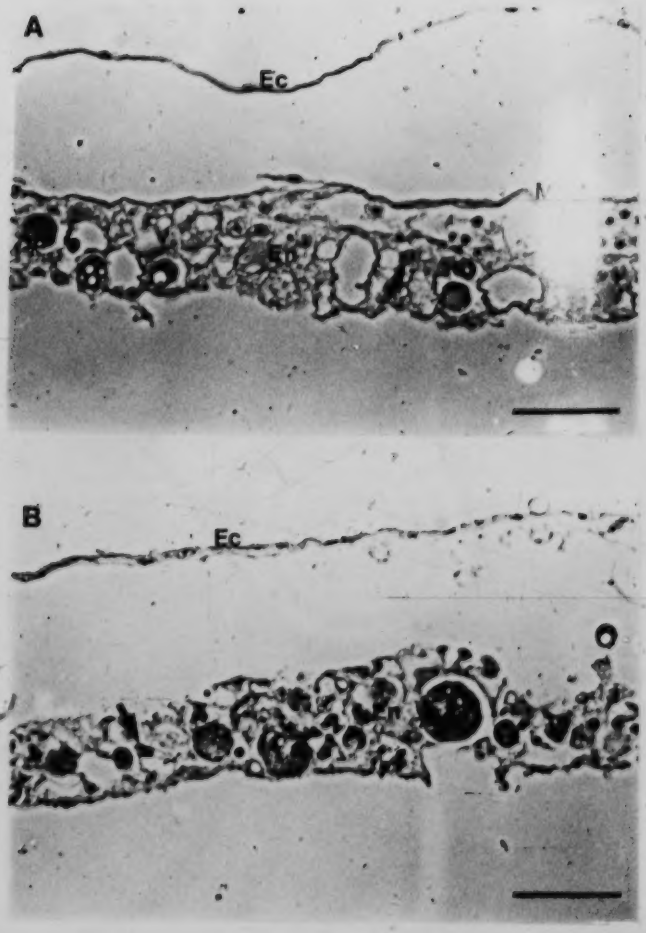


Figure 8.

Light micrographs of representative sections through the area vasculosa (A) and the area vitellina (B) from the yolk sac of a 3-day chick embryo. The ectoderm (Ec), mesoderm (M) with blood vessels and endoderm (En) can be clearly seen in these sections. Endodermal nuclei (arrows) and yolk granules (*) can be seen in the endoderm of both A and B. The bar is 50 μm.

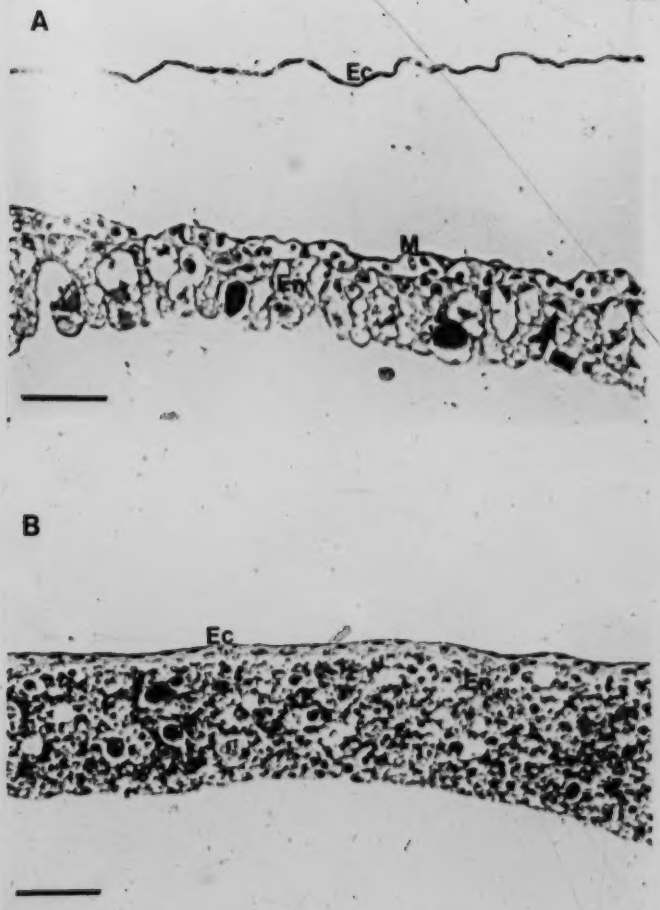


Figure 9.

Light micrographs of representative sections through the area vasculosa (A) and the area vitellina (B) from the yolk sac of a 4-day chick embryo. The cell layers are ectoderm (Ec), mesoderm (M) containing blood islands and endoderm (En) containing yolk granules (*). Endodermal nuclei (arrow) can be clearly seen in A. The bar is 50 μm.

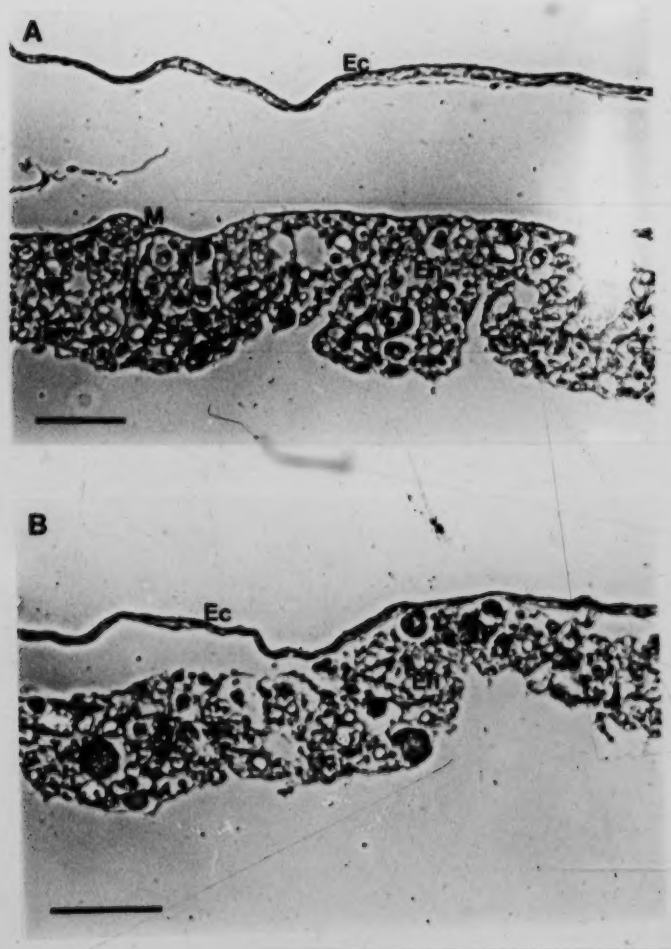


Figure 10.

Light micrographs of representative sections through the area vasculosa (A) and the area vitellina (B) from the yolk sac of a 5-day chick embryo. Observe the ectoderm (Ec), the mesoderm (M) in A only and the endoderm (En) which contains yolk granules (*) and in which nuclei (arrow) can be seen. The bar is 50 μm.

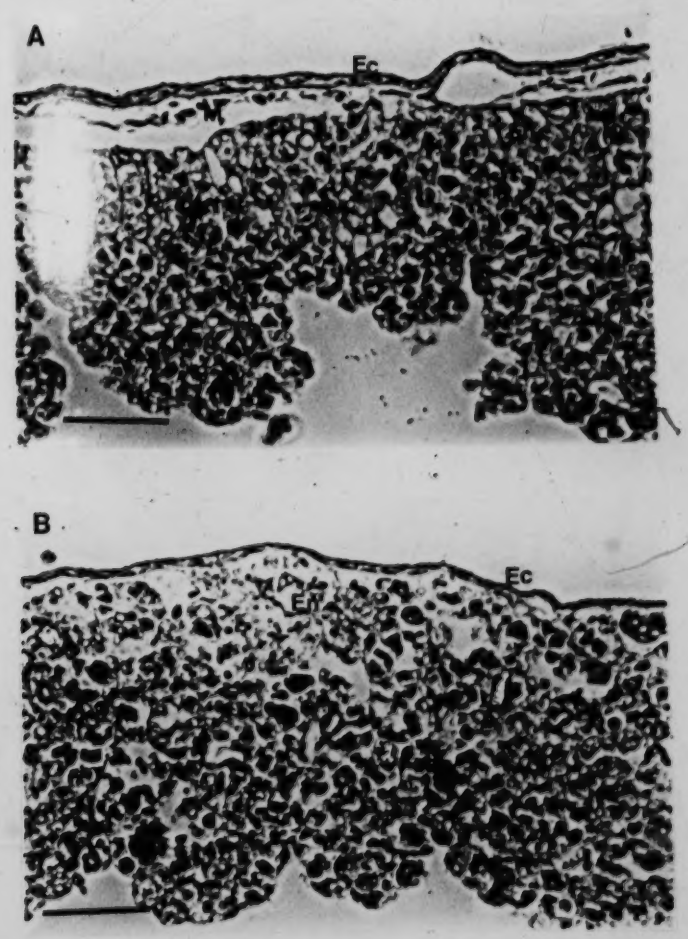


Figure 11.

Light micrographs of representative sections through the area vasculosa (A) and the area vitellina (B) from the yolk sac of a 7-day chick embryo. The cell layers are ectoderm (Ec), mesoderm (M) with blood vessels and endoderm (En). Numerous yolk inclusions (*) can be seen in the latter cell layer. Endodermal nuclei (arrow) can be observed dispersed between the yolk inclusions. The bar is 50 μm.

latter cell layer is usually observed adhering to the endodermal layer and it is conspicuous because of the presence of the blood vessels, which form within it. This layer consists of flattened cells. On the other hand, the somatic mesoderm was only occasionally seen adhering to the undersurface of the ectoderm and it is also a layer of flattened cells. The endodermal layer of the area vasculosa is morphologically more differentiated than that of the area vitellina and is usually composed of columnar cells organized as a monolayer. The cells are closely packed and contain many yolk granules which make it difficult to discern cell boundaries. At the earliest stages of development these cells are relatively small and at later stages of development they increase in height and contain numerous yolk inclusions.

Sections taken from within the area vitellina show two cell layers, the ectoderm and the endoderm (Figures 7B, 8B, 9B, 10B, 11B). The ectodermal cells, at progressively later stages of development, generally retain a monolayered arrangement, although in some cases a bilayered arrangement can be observed. The endoderm in this region is less differentiated than that of the area vasculosa and is composed of several layers of cells with many yolk inclusions. These cells are loosely packed in an irregular manner and are separated from each other, by extracellular yolk granules such that the plasma membrane boundaries are not distinguishable. At the later stages of development, the yolk inclusions are diminished in size and so numerous that cell membrane boundaries are also not distinguishable.

2. Extraction of Lectin Activity

A first approach to the problem of isolating lectin activity from a. vit. and a. vasc. tissue of stage 13-15 embryos was an investigation of the effect of different components of the extraction solution on extractability and preservation of lectin activity; this has not been done previously. The lectin extraction solution (LES) used in extracting β-D-galactoside-binding lectins from primitive streak stage chicken embryos (Zalik et al., 1983), was used in initial experiments. In addition to this complete LES, three other extraction solutions were used; LES (-lactose), LES (-2ME) and LES (-EDTA, + CA^{++} and Mg^{++}). The mean lectin titer values (HU/ml) for a. vit. and a. vasc. tissue extracted with complete LES were taken as a standard and the values obtained with the other three extraction solutions were expressed as the percent of these standard values. Tissue from about 25 to 35 a. vit. and a. vasc. were extracted in each experiment and three separate determinations of lectin titer were made for each of the four extraction solutions. In each experiment, a. vit. and a. vasc. tissue were prepared simultaneously and both extracts were assayed at the same time. Lectin activity was assayed in the same extract immediately after extraction, (T_o) and after four months at -20°C, (T_{4mo}). The results of this investigation are presented in Table 1. Higher lectin activities were usually observed in extracts obtained from tissue of the a. vasc. as compared with extracts of the a. vit.

Because of the limited amount of tissue available different sets of embryos were used in each experiment. In addition different

Table 1.

Effect of different components of the extraction solution on the extractability and preservation of crude lectin activity. Tissues were dissected, washed and homogenized in extraction solution at a ratio of 1:10 (packed tissue mass:volume of extraction solution). Lectin activity was assayed in the same extract immediately after extraction (T_o) and after 4 months of storage at -20°C (T_{4mo}). In each extraction solution, three different experiements each with a different set of embryos was performed. Area vitellina and area vasculosa tissue were extracted and assayed simultaneously.

Table 1.

Extraction Solutions	Expt No.	Titre (HU/ml)			
		Area Vitellina		Area Vasculosa	
		T_o	T_{4mo}	T_o	T_{4mo}
LES[a] complete	1	1920	5120	3840	10240
	2	1280	1280	7680	7680
	3	640	1280	5120	10240
		1280 ± 370[b]	2560 ± 1280	5547 ± 3202	9387 ± 5420
		10.18 ± 0.46[e]	10.99 ± 0.67	12.38 ± 0.29	13.18 ± 0.14
LES (-Lactose)	4	1280	640	2560	2560
	5	640	40	2560	640
	6	320	0	2560	5120
		747 ± 282 58%[c]	227 ± 207 8% (30%)[d]	2560 ± 0 46%	2773 ± 1601 29%
		9.32 ± 0.58	4.88 ± 2.7	11.32 ± 0	10.99 ± 0.88
LES (-2ME)	7	960	0	5760	2560
	8	2560	640	5120	2560
	9	640	640	5120	1280
		1387 ± 594 108%	427 ± 213 16% (30%)	5333 ± 213 96%	2133 ± 427 23% (40%)
		10.18 ± 0.59	6.22 ± 3.11	12.38 ± 0.06	10.99 ± 0.33
LES (-EDTA	10	640	1280	2560	5120
+Ca	11	10240	640	10240	2560
+Mg)	12	5120	1280	10240	5120
		5333 ± 3079 416%	1067 ± 213 42% (20%)	7680 ± 2560 138%	4267 ± 853 45% (55%)
		11.65 ± 1.2	9.99 ± 0.33	12.65 ± 0.67	11.99 ± 0.33

Table 1 (Continued)

[a]Lectin extraction solution consisting of 0.15 M NaCL, 0.005 M phosphate buffer, 0.002 M EDTA, 0.004 M 2ME, containing 0.3M lactose, 0.25 mM PMSF and 0.02% NaN_3 pH 7.2.

[b]In all columns, this value represents the mean and standard error of three experiments.

[c]In all columns, this value represents the percent of the mean lectin titre obtained with complete LES

[d]Values represent lectin activity remaining in extracts after T_{4mo}, when compared to activity assayed at T_o.

[e]In all columns, this value represents the mean and standard error of individual titer values converted to $\log_2$. This is performed because the dilution of the extract is doubled in the process of serially diluting it in agglutination saline.

preparations of test erythrocytes were used to assay activity. These factors and the semiquantitative nature of the haemagglutination assay may account for the variability of the data obtained in repeat experiments.

In Table 1 it can be seen that lectin activity increased or remained constant in extracts obtained with complete LES, after being stored at -20°C. Extracts of tissues obtained in the absence of lactose, LES (-lactose), showed a reduction in lectin activity; a. vit. extracts were 58% and a. vasc. extracts were 46% of the values obtained with complete LES. After 4 months, the titer in extracts of the a. vit. was only 30% of that of the original extract, while that of the a. vasc. did not change noticeably. When compared to extracts obtained with complete LES and maintained for 4 months, lectin activity was much lower, 8% and 29% in the a. vit. and a. vasc. respectively.

Tissues extracted in the absence of 2ME, LES (-2ME), had similar activities to those in complete LES, however the preservation of lectin activity was not as efficient. In the a. vit. the lectin activity of stored extracts was 30% of the original extracts, while in stored extracts of the a. vasc. only 40% of the lectin activity remained. When compared to extracts stored in complete LES, those stored in absence of 2ME had only 16% of lectin activity, for the a. vit., while in extracts of the a. vasc. only 23% of the lectin activity remained.

Lastly, in the presence of Ca^{++} and Mg^{++} (LES - EDTA, $+Ca^{++}$ and Mg^{++}) initial extraction of lectin activity appeared to be enhanced when compared to complete LES extracts, but the preservation of lectin activity was not as efficient. In this case, after four months the

lectin titers were only 20% and 55% of the original extract, for a. vit. and a. vasc. respectively. And extracts stored in the presence of Ca^{++} and Mg^{++} had 42% and 45% of lectin activity of extracts stored in complete LES, for the a. vit. and a. vasc. respectively.

These results show that the components of the extraction solution necessary for efficient extraction of lectin activity and preservation of this activity over time, include lactose, 2ME and EDTA. The addition of lactose to the extraction solution facilitated the extraction of lectin activity from both tissues. This component was also required for the preservation of activity. The sulphydryl reducing agent, 2ME was also an important extraction solution constituent for the preservation of activity, as was the absence of Ca^{++} and Mg^{++} ions. On the basis of these preliminary experiments, complete LES containing lactose, 2ME and EDTA was selected for use in all subsequent extraction procedures.

3. Specific Activity of Crude Lectin Extracts from Several Developmental Stages

Following the detection of haemagglutination activity in crude, soluble extracts of tissues from the a. vit. and a. vasc. of embryos at stage 13-15, an attempt was made to determine if changes in lectin activity could be detected in these tissues at subsequent developmental stages. Some endogenous chicken lectins, such as that from embryonic pectoral muscle, have been shown to be developmentally regulated (Nowak et al., 1976). It was felt that an investigation of possible

developmental regulation of lectin from developing yolk sac tissue would be of value in elucidating its function.

The stages of development studied in addition to stage 13-15 (2d) embryos, were stage 18 (3d), stage 22 (4d), stage 26 (5d) and stage 30 (7d) embryos. Between four and ten embryos were used in these experiments. In each case, separate determinations of specific activity from three different extracts of a. vit. and a. vasc. tissue were made. The results of these determinations are shown in Table 2.

In all of the extracts examined the specific activities of the lectin obtained from a. vasc. tissue extracts were significantly higher than those from a. vit. extracts, as judged by an analysis of variance ($p<0.01$). The differences between the specific activities of the five different stages were then examined by a Least Significant Difference test. The specific lectin activity was significantly different in extracts of 2 day, 3 day, 4 day and 5 day tissues ($p<0.05$). The differences in specific activity between 5 day and 7 day extracts were not significant.

4. Effect of Different Saccharides on Haemagglutination Activity

The effects of different saccharides on haemagglutination activity was determined in extracts from tissues of the a. vit. and a. vasc. of stage 13-15 embryos. This was a necessary first step in the selection of an appropriate saccharide ligand for the preparation of an affinity chromatography column for lectin purification, based on ability of the lectin to bind saccharides specifically and reversibly. It was also important to eludicate the saccharide-binding specificity of the lectins

Table 2.

Specific activities (HU/mg protein) of crude lectin extracts from different developmental stages. In all experiments tissues were dissected, washed and homogenized in extraction solution at a ratio of 1:10 (packed tissue mass:volume of extraction solution). After dialysis at 4°C, extracts were titrated and their protein concentrations were determined as described in the text. For each developmental stage, three experiments were carried out each with a different set of embryos. Activity was assayed simultaneously in area vitellina and area vasculosa extracts.

For statistical analysis, the individual values for specific activities were converted to the $\log_2$ (Finney et al., 1955) and subjected to an analysis of variance. When this was performed the differences between the two areas as well as between the five stages were significant ($p<0.01$). The differences in specific activities between the stages were then examined by a Least Significant Difference test and were found to be significant ($p<0.05$) between 2 day, 3 day, 4 day and 5 day extracts. The values between 5 and 7 days were not significant.

Table 2.

Stage	Expt. No.	Specific Activity (HU/mg protein)			
		Area Vitellina		Area Vasculosa	
			Log_2		Log_2
13–15 (2 days)[a]	1	539	9.074	3151	11.621
	2	1028	10.009	2926	11.517
	3	1180	10.205	4935	12.270
			9.763 ± 0.349[b]		11.803 ± 0.236
18 (3 days)	4	2639	11.366	6059	12.565
	5	1900	10.892	5705	12.477
	6	1177	10.205	7516	12.877
			10.821 ± 0.337		12.64 ± 0.121
22 (4 days)	7	3122	11.607	8533	13.058
	8	2901	11.502	6827	12.738
	9	6073	12.568	9910	13.275
			11.892 ± 0.339		13.024 ± 0.156
26 (5 days)	10	9470	13.207	10708	13.385
	11	4708	12.202	7938	12.955
	12	6827	12.738	20480	14.323
			12.716 ± 0.29		13.554 ± 0.404
30 (7 days)	13	4995	12.288	12457	13.610
	14	15754	13.948	13213	13.688
	15	3821	11.899	21113	14.365
			12.712 ± 0.628		13.888 ± 0.240

[a]Days of incubation

[b]In all columns, this value represents the mean and standard error of three experiments in which the individual specific activity values were converted to Log_2

isolated in this study, because of the importance of this property to the biological activity of these macromolecules. The saccharide-binding specificity of crude extracts of 16-day chicken embryonic pectoral muscle was also determined for comparative purposes. This tissue source has been previously shown to contain soluble β-D-galactoside-binding lectin activity (Nowak et al., 1976).

The saccharide-specificity of lectin extracts from the a. vit. and a. vasc. of stage 13-15 embryos as well as of 16-day pectoral muscle is shown in Table 3. In this table, the saccharide concentration (mM) necessary to inhibit at least 4 HU of lectin is presented. The three crude lectin extracts showed similar results, since the three most potent inhibitors of haemagglutination activity in order of decreasing potency were the same; TDG [β-D-gal (1-->1) β-D-thiogal], lactose [β-D-gal (1-->4) glc] and RGG [(2R)-Glycerol-O-β-D-galactopyranoside]. Of the three sources of lectin, a. vit. extracts were inhibited by the lowest concentration of saccharides; 0.16 mM, 0.56 mM and 3.18 mM for TDG, lactose and RGG, respectively. Extracts from the a. vasc. were inhibited by 0.25 mM, 0.66 mM and 8.38 mM for TDG, lactose and RGG, respectively. And for embryonic pectoral muscle the concentrations of these saccharides were 0.29 mM, 1.0 mM and 12.5 mM, for TDG, lactose and RGG respectively.

Other weaker inhibitors of these lectins were isopropyl-β-D-thiogalactopyranoside, methyl-α-D-galactopyranoside, phenyl-β-D-galactopyranoside, galactose, methyl-β-D-galactopyranoside and α-D-

Table 3

Effect of different saccharides on the haemagglutination activity of crude extracts of stage 13-15 area vitellina and area vasculosa and 16-day embryonic pectoral muscle tissues. Extracts were diluted to a titer of either 8 or 4 haemagglutinating units (HU). Each saccharide was used at decreasing concentrations to determine which of these reduced the titer from 8 to 4 HU or abolished 4 HU completely. The lowest concentration of saccharide with inhibitory activity was recorded. Values represent the mean of 4 determinations, each obtained with an extract from a different set of embryos. For each sugar four to five different concentrations were tested (see page 42).

Table 3.

Saccharide	Concentration (mM) Area Vitellina	Area Vasculosa	Pectoral Muscle
Thiodigalactoside	0.16 ± 0.05[a]	0.25 ± 0.00	0.29 ± 0.04
Lactose	0.56 ± 0.02	0.66 ± 0.20	1.00 ± 0.00
(2R)-Glycerol-O-β D-Galactopyranoside	3.18 ± 2.90	8.38 ± 2.50	12.50 ± 0.00
Isopropyl-β-D-thio galactopyranoside	20.00 ± 11.30	34.00 ± 9.40	38.00 ± 7.20
methyl-α-D-galactopyranoside	18.00 ± 10.80	38.00 ± 7.20	34.00 ± 9.90
phenyl-β-D-galactopyranoside	39.00 ± 11.20	38.00 ± 7.20	44.00 ± 6.20
methyl-β-D-galactopyranoside	32.00 ± 23.10	62.00 ± 21.60	81.00 ± 18.80
Galactose	36.00 ± 16.00	56.00 ± 12.00	75.00 ± 00.00
α-D-melibiose	78.00 ± 22.50	62.00 ± 12.50	75.00 ± 14.40
methyl-α-D-mannopyranoside	>100.00	>100.00	>100.00
N-Acetyl-D-galactosamine	>100.00	>100.00	>100.00
N-Acetyl-D-glucosamine	>100.00	>100.00	>100.00
L-fucose	>100.00	>100.00	>100.00
D-fucose	>100.00	>100.00	>100.00

[a]In all columns, this value represents the mean and standard error of four experiments

melibiose. The relatively higher inhibitory capacity of methyl-α-D-galactopyranoside as compared to its β-anomer is not understood, but has been reported previously (Nowak et al., 1977; Zalik et al., 1983). Several other monosaccharides tested including GlcNAc, GalNAc, methyl-α-D-mannopyranoside, L-fucose and D-fucose did not inhibit haemagglutination activity at concentrations up to 100 mM, the highest tested. The concentrations of saccharides needed to inhibit crude lectin extracts from 16-day pectoral muscle were somewhat higher than those reported by Nowak et al. (1976), for this tissue.

5. Purification of the Soluble Lectin by Affinity Chromatography

Extracts from tissue of approximately 190-220 area vitellinae and area vasculosae of stage 13-15 embryos and from 4-5 pectoral muscles from 16-day embryos, were used in each purification experiment. These extracts were purified by affinity chromatography on a column bearing the ligand ρ-aminophenyl-β-D-Lactoside (APL). Lectin activity was eluted with 0.3 M lactose and the elution patterns of the three lectins were very similar (see Figure 12). No detectable activity was eluted with 0.3 M sucrose, thus it would appear that these lectins bound specifically and reversibly to the terminal galactosyl groups of APL.

A summary of the purification of soluble lectin from the three tissues is presented in Table 4. Values reported here represent the means and ranges of six separate determinations for a. vit. and a. vasc. lectins and one determination for the muscle lectin. The specific activities of crude lectin extracts were quite variable, ranging from 156 to 900 HU/mg protein for a. vit. extracts and from 1670 to 7314

Figure 12.

Affinity purification of soluble lectin extracts from area vasculosa (a) and area vitellina (b) tissue of stage 13-15 embryos and from pectoral muscle tissue (c) of 16-day embryos on an APL-Sepharose 4B column. At the point indicated by: Arrow 1, 0.3M sucrose in MEPBS was added; at Arrow 2, MEPBS was added and at Arrow 3, 0.3M lactose in MEPBS was added. Extracts were obtained from 190 to 220 area vitellinae and area vasculosae tissue and 4 to 5 pectoral muscle tissue.

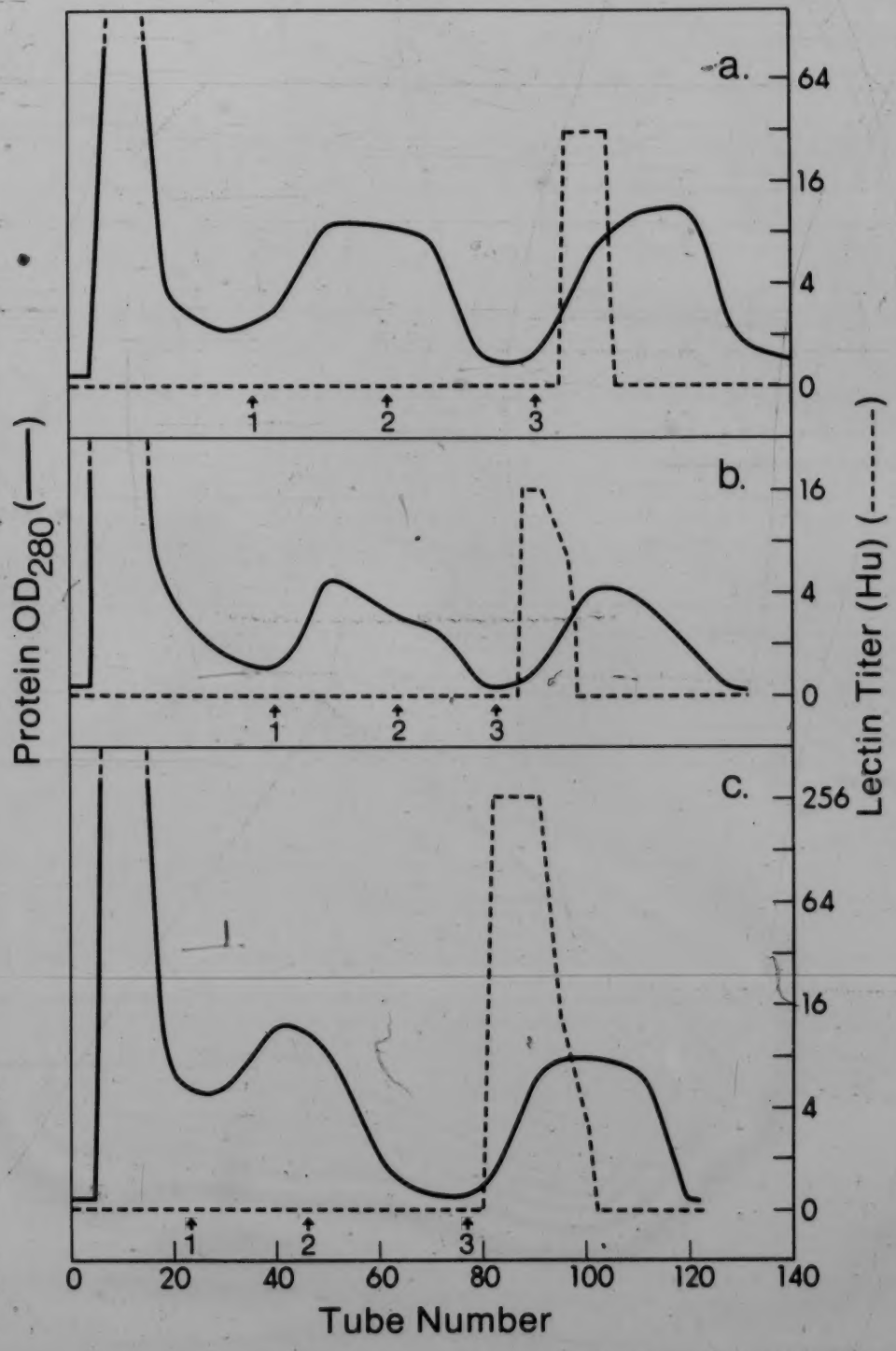

a.
b.
c.
64
16
4
0
256
1
2
3
Protein OD_{280} (——)
Lectin Titer (Hu) (------)
0
20
40
60
80
100
120
140
Tube Number

Table 4.

Summary of the purification of soluble, β-D-galactoside-binding lectins from area vitellina and area vasculosa tissue of stage 13–15 embryos and from 16-day embryonic pectoral muscle tissue by affinity chromatography on an APL-Sepharose column. Values represent the means and ranges of six determinations. Extracts from 190 to 220 stage 13–15 embryos and 4 to 5, 16-day muscle tissue were normally used.

Table 4

	Activity	Protein		
	Specific Activity HU/mg protein	Total (mg) present in extract	% of total soluble protein present in pure lectin fractions	Recovery (%)
Area Vitellina				
Crude extract	558	57.4		
	(156 - 900)[a]	(47.4 - 70.6)		
Pure fraction	2.23×10^5	0.096	0.2	52
	(9.9×10^4 - 5.4×10^5)	(0.041 - 0.16)	(0.06 - 0.34)	(20 - 75)
Area Vasculosa				
Crude extract	3516	22.8		
	(1670 - 7314)	(17.5 - 37.9)		
Pure fraction	9.26×10^5	0.066	0.3	29
	(6.07×10^4 - 2.91×10^6)	(0.022 - 0.176)	(0.12 - 1.0)	(11 - 60)
Pectoral Muscle[b]				
Crude extract	54,613	24		
Pure fraction	7.8×10^5	0.186	0.8	60

Table 4 (Continued)

[a]In all columns, this value represents the range of six determinations

[b]Only one determination was made

HU/mg protein for a. vasc. extracts. The muscle extract had a specific activity of 54,613 HU/mg protein. After purification specific activities ranged from 9.9×10^4 to 5.4×10^5 HU/mg protein for a. vit. and from 6.07×10^4 to 2.91×10^6 HU/mg protein for a. vasc. Purified lectin from the pectoral muscle had a specific activity of 7.8×10^5 HU/mg protein. Although the specific activities of the purified chick lectins were quite variable, in general higher levels were found in the a. vasc. preparations, 9.26×10^5 HU/mg protein (mean specific activity) as compared to the a. vit. preparation, 2.23×10^5 HU/mg protein (mean specific activity).

6. Saccharide Specificity of Purified Lectin

Purified lectin extracts were tested for saccharide specificity. Four separate determinations were made for purified lectin preparations from a. vit. and a. vasc. and one determination was made for the muscle lectin. The results of these assays are reported in Table 5 (along with the results for crude extracts for comparative purposes).

As was observed for crude extracts, the three most potent inhibitors of lectin activity were TDG, lactose and RGG. In general, somewhat higher saccharide concentrations were necessary to inhibit the purified lectin from a. vit. tissue as compared to the results for crude lectin extracts. However, for the a. vasc. tissue, lower concentrations of the same saccharides were found to inhibit the purified lectin preparations as compared to crude lectin extracts. Higher saccharide concentrations were also needed to inhibit purified muscle lectin. The saccharide specificity of the three purified lectins was the same as

Table 5.

Effect of different saccharides on the haemagglutination activity of pure and crude lectin obtained from tissue of the area vitellina and area vasculosa of stage 13-15 embryos and from the pectoral muscle of 16-day embryos. Extracts were diluted to 8 or 4 haemagglutinating units (HU). Each sugar was used at decreasing concentrations to determine which of these reduced the titre from 8 to 4 HU or from 4 to 0 HU. The lowest concentration of saccharide with inhibitory activity was recorded. Values represent mean concentrations (mM) of four different determinations except for purified muscle lectin which was assayed only one time. Four to five different concentrations were tested for each sugar (see page 42).

Table 5

Saccharide	Area Vitellina		Area Vasculosa		Pectoral Muscle	
	pure[a]	crude[a]	pure[a]	crude[a]	pure[b]	crude[a]
Thiodigalactoside	0.20 ± 0.05[a]	0.16 ± 0.05	0.14 ± 0.06	0.25 ± 0.00	0.4	0.29 ± 0.04
Lactose	1.00 ± 0.02	0.56 ± 0.02	0.41 ± 0.20	0.66 ± 0.20	1.0	1.00 ± 0.00
(2R) Glycerol-O-β-D-galacto-pyranoside	7.80 ± 2.90	3.18 ± 2.90	4.12 ± 2.80	8.38 ± 2.50	12.5	12.50 ± 0.00
Isopropyl-β-D-thiogalacto-pyranoside	50.00 ± 0.00	20.00 ± 11.30	16.25 ± 12.00	34.00 ± 9.40	50.0	38.00 ± 7.20
Methyl-α-D-galactopyranoside	44.00 ± 6.00	18.00 ± 10.80	17.50 ± 4.30	38.00 ± 7.20	50.0	34.00 ± 9.90
phenyl-β-D-galactopyranoside	38.00 ± 12.00	39.00 ± 11.20	31.20 ± 6.20	38.00 ± 7.20	50.0	44.00 ± 6.20
Methyl-β-D-galactopyranoside	100.00 ± 0.00	32.00 ± 23.10	20.00 ± 5.00	62.00 ± 21.60	100.0	81.00 ± 18.80
Galactose	52.00 ± 15.00	36.00 ± 16.00	27.50 ± 8.30	56.00 ± 12.00	75.0	75.00 ± 0.00
α-D-melibiose	76.00 ± 24.00	78.00 ± 22.50	40.00 ± 20.00	62.00 ± 12.50	100.0	75.00 ± 14.40
Methyl-α-D-manno pyranoside	>100.00	>100.00	>100.00	>100.00	>100.0	>100.00

Table 5, continued

Saccharide	Area Vitellina		Area Vasculosa		Pectoral Muscle	
	pure[a]	crude[a]	pure[a]	crude[a]	pure[b]	crude[a]
N-acetyl-D-galactosamine	>100.00	>100.00	>100.00	>100.00	>100.0	>100.00
N-acetyl-D-glucosamine	>100.00	>100.00	>100.00	>100.00	>100.0	>100.00
L-fucose	>100.00	>100.00	>100.00	>100.00	>100.0	>100.00
D-fucose	>100.00	>100.00	>100.00	>100.00	>100.0	>100.00

[a]Mean and standard error of 4 experiments

[b]Concentration (mM) of one determination

that for the crude extracts, with an affinity for saccharides bearing a β-D-galactoside configuration. However of the three lectins, purified lectin from the a. vasc. required the lowest concentrations of saccharides bearing a D-galactoside configuration, to be inhibited. The increase in affinity of lectin for the saccharides, TDG, lactose and RGG over crude extracts suggests that some contaminating material affecting the affinity of the lectin may have been removed during the purification step. Also, in purified lectin of the a. vasc. the differences in affinity between methyl-α- and methyl-β-D-galactopyranoside was no longer obvious. Other weaker inhibitors of the a. vasc. lectin include isopropyl-β-D-thiogalactopyranoside, galactose, phenyl-β-D-galactopyranoside and α-D-melibiose. For lectin purified from the a. vit., the increase in saccharide concentrations needed to inhibit activity compared to crude extracts suggests some loss in affinity. The specific activity of purified lectin preparations from this tissue source was one-quarter that of the area vasculosa (Table 4), which may indicate the presence of contaminating material in this extract. As reported previously for crude lectin preparations, methyl-α-D-galactopyranoside seemed to be a better inhibitor of the purified lectin than its β-anomer. The monosaccharides methyl-α-D-mannopyranoside, GalNAc, GlcNAc, L-fucose and D-fucose again showed no inhibitory effect on the purified lectins from these three tissues at the concentrations tested. This suggests that these lectins do not have an appreciable affinity for the above saccharides.

The inhibition of purified lectin from a. vit. and a. vasc. tissue extracts by the saccharides TDG and lactose are comparable to results obtained for the soluble lectin ($LI_{(s)}$) and the particle associated lectin ($LII_{(p)}$) purified from primitive streak stage embryos (Zalik et al., 1983). The concentrations of TDG and lactose needed to inhibit purified lectin extracts from pectoral muscle were higher than those reported by Nowak et al. (1977). However since these workers reported the concentrations of sugar needed to inhibit haemagglutination by 50% these results are not strictly comparable to those reported here.

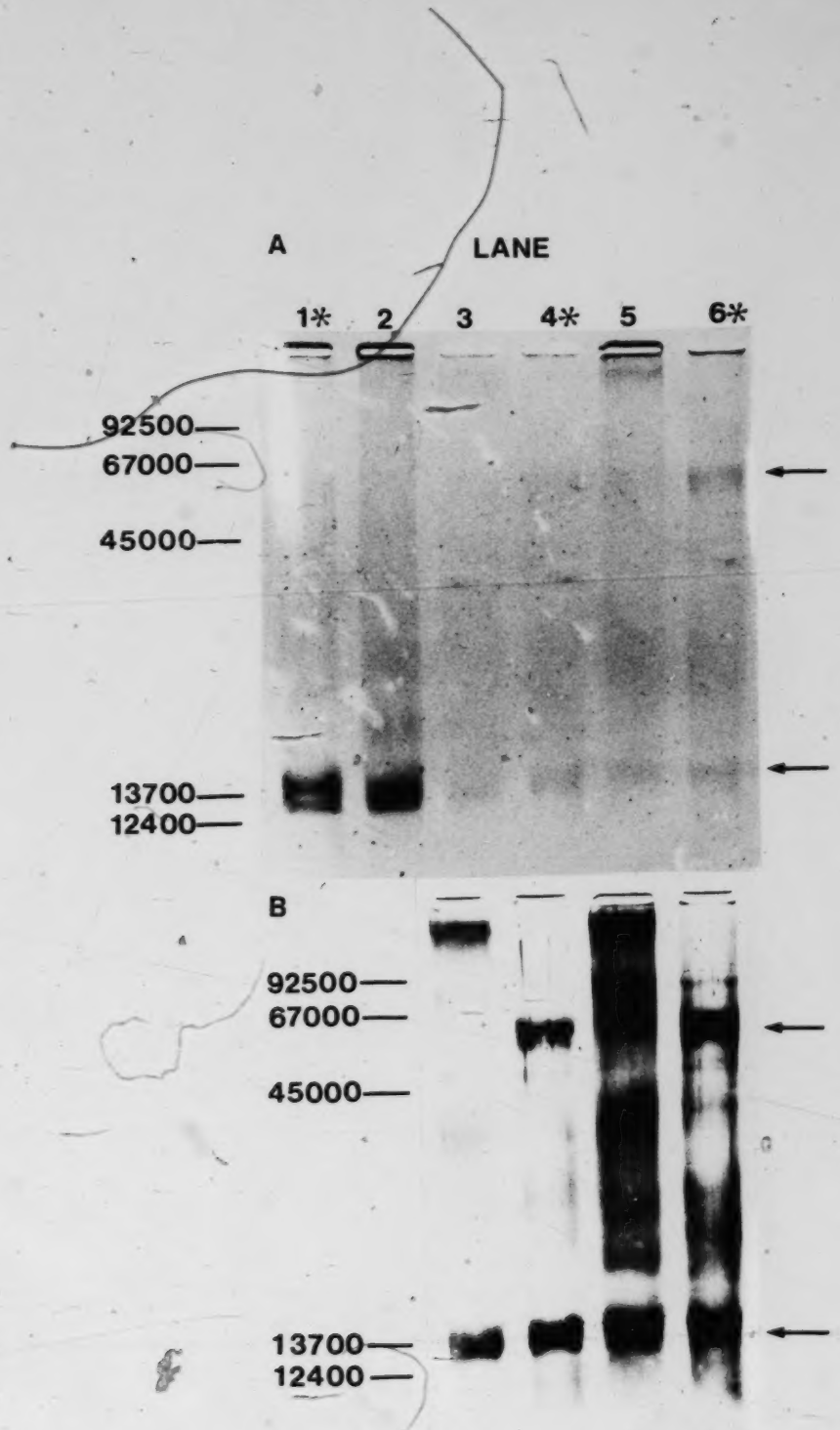
7. Subunit Molecular Weight Determinations

Purified lectin preparations of area vitellina, area vasculosa and pectoral muscle were examined by SDS-polyacrylamide gel electrophoresis (Figure 13). Lectin protein concentrations of 3-14 μg total for a. vit. and a. vasc. preparations and about 12 μg for muscle lectin were normally used. Gels were stained with Coomassie blue and subsequently with a silver stain. Staining of protein bands in Coomassie blue was generally very faint probably because there was insufficient protein for detection by this dye. The use of the silver staining method after Coomassie blue staining generally resulted in better visualization of the protein bands (see Figure 13), and in general bands observed in Coomassie blue were also found on silver staining.

Lyophilized protein samples from a. vit. and a. vasc. tissue solubilized in buffer containing SDS, urea and 2ME (reducing conditions) gave two bands (see Figure 13). The molecular weights of these bands were estimated to be 68,100 ± 700 (mean ± SE) and 14,200 ± 100 for a.

Figure 13.

SDS-polyacrylamide gel electrophoresis of purified lectin extracts from area vitellina and area vasculosa tissue of stage 13-15 embryos and from pectoral muscle of 16-day embryos. A representative gel stained with Coomassie blue (A) and silver (B) is shown. Samples were solubilized under reducing (2% SDS, 8M urea and 5% mercaptoethanol) and non-reducing (2% SDS) conditions. Lanes with samples in reducing conditions are indicated by an asterisk (*). Lanes 1 and 2, pectoral muscle lectin; Lanes 3 and 4, area vasculosa lectin; Lanes 5 and 6, area vitellina lectin. Arrows point to the high molecular weight protein component under reducing conditions and the low molecular weight protein component under both reducing and non-reducing conditions for the area vasculosa and area vitellina lectins. Standards were: Phosphorylase B (92,500); BSA (67,000); ovalbumin (45,000); ribonuclease A (13,700) and cytochrome C (12,400).


A
LANE
1*
2
3
4*
5
6*
92500
67000
45000
13700
12400
B
92500
67000
45000
13700
12400

vit. lectin preparation, based on five independent studies using two different purified batches of material. And for the a. vasc. lectin preparations the molecular weights were 68,700 ± 1,200 and 13,700 ± 300, based on ten independent studies using five different purified extracts. In some gels, the lectin preparation from a. vit. gave a high molecular weight doublet, with molecular weights of 71,300 ± 1,300 and 62,700 ± 1,400 as opposed to a single band. This doublet was observed in four separate gels using three different extracts. However it was never observed in a. vasc. lectin preparations analyzed in the same gels. A probable reason for the appearance of the doublet was due to the separation of the generally broad, dark staining band recorded as a single band in silver stained gels. From these studies it was determined that the affinity-purified lectin preparations from the a. vit. and a. vasc. consist of at least two protein components.

When lyophilized lectin preparations were solubilized in buffer containing only SDS (non-reducing conditions), a low molecular weight band similar to that reported above appeared consistently with a molecular weight of about 13,400 ± 300 for a. vit. lectin and 13,400 ± 200 for a. vasc. lectin preparations. In the a. vit. lectin preparation, a new band not seen previously, with a molecular weight of about 127,000 ± 2,000 was observed in four independent studies using two different extracts. For the a. vasc. lectin, this band had a molecular weight of about 124,000 ± 6,700 and it was observed in two of five independent studies using two different extracts (Figure 13).

The pectoral muscle lectin had a molecular weight of about 14,300 ± 200 in three independent experiments using one purified extract. A minor band, with a molecular weight of about 12,700 ± 200 was also observed in these gels. These results were obtained when lectin was solubilized in buffer containing SDS, urea and 2ME and they agree with those reported previously (Nowak et al., 1977). On the other hand when lectin was solubilized in buffer containing only SDS, one band with an estimated molecular weight of 12,800 ± 400 was observed (Figure 13).

8. Analysis of Lectin Activity by Gel Filtration Chromatography

To determine the molecular configuration of the lectin in aqueous solution, crude extracts from a. vit. and a. vasc. tissues were analyzed by gel filtration chromatography on a Sephacryl column, under non dissociating conditions. The column was calibrated with a series of standards in the molecular weight range from 67,000 (BSA) to 12,400 (cytochrome C). The extracts were examined in the presence of lactose, in three separate determinations.

Haemagglutination activity eluted as a continuum in the molecular weight range from 56,000 to 35,000 for a. vit. extracts and from 65,400 to 10,000 for a. vasc. extracts. In the latter extract a peak of activity eluted just behind ovalbumin with a molecular weight of about 41,000. This was determined from the fraction with the highest lectin titer. However most of the lectin activity was detected in a large trailing tail with molecular weights down to about 10,000. Recovery of activity from the column was very low which could be as a result of inactivation of lectin activity or to non-specific association of the

lectin with the Sephacryl beads. Due to these difficulties, the results of this investigation were inconclusive.

9. Lectin Characterization by Isoelectric Focusing

Purified lectin preparations from a. vit. and a. vasc. tissue were analyzed by isoelectric focusing in thin layer polyacrylamide gels and stained with Coomassie blue followed by silver stain. These preparations were found to be heterogeneous as was expected from the results with SDS-polyacrylamide gel electrophoresis.

In the purified extract from a. vit. tissue, a major band with a pI value of about 6.4 ± 0.4 (mean ± SE) was observed in two gels of six that were stained with Coomassie blue. In addition, two minor bands with pI values of 5.0 and 5.2 which stained very faintly were observed in only one gel. The a. vasc. purified lectin appeared as a band with an approximate isoelectric point of 6.8 when stained with Coomassie blue. This band was observed only once. The lack of positive staining in this stain is probably due to low protein concentrations because many more bands were observed when gels were stained with silver. When gels were examined after silver staining, a main band with pI 6.64 ± 0.16 in addition to several minor bands in the acidic pI range from 4.1 to 5.5 were observed for a. vit. lectin extracts. Lectin preparations from a. vasc. tissue gave similar results; a main band with a pI value of about 6.61 ± 0.13 as well as several bands between pI values of 4.05 and 5.1 were observed.

In summary, the most consistently stained band in both Coomassie blue and silver had an average pI value of 6.58 ± 0.14 for a. vit. lectin preparations. Two to four minor bands were observed between pI values of 4.1 and 5.5. For a. vasc. lectin preparations, the average pI was 6.65 ± 0.11 for the main band and two to four minor bands were observed in the acidic pI range 4.05 to 5.1. Because of the variability in the staining patterns these results were considered inconclusive.

DISCUSSION

This study has focused on the isolation and characterization of soluble, endogenous lectin activity from the area vitellina and area vasculosa tissue of the developing yolk sac of the chick embryo. Soluble proteins extracted from these tissues agglutinated trypsinized rabbit erythrocytes in a haemagglutination assay used to detect and quantitate lectin activity. The specific activity of the lectin(s) extracted from the area vasculosa tissue of the yolk sac was significantly higher than that from the area vitellina tissue in all of the extracts that were examined. Lectin activity extracted from 2-day area vitellina and area vasculosa tissue was purified and characterized. The preparations from both tissues appear to be very similar by all criteria tested, including saccharide specificity, subunit molecular weight and isoelectric point, suggesting that the lectin(s) in these two tissues is similar, if not identical.

Several characteristics of these proteins were determined in this study. The endogenous haemagglutination activity from area vitellina and area vasculosa tissue is extractable in the presence of lactose, 2-mercaptoethanol and EDTA. And these conditions are the most useful for the extraction and preservation of this activity. Lectin preparations from both areas have similar saccharide-binding affinities, reacting with saccharides bearing a β-D-galactoside configuration. The specificity is defined in terms of the most potent inhibitors of lectin

activity which were thiodigalactoside [β-D-gal(1-->1) β-D-thiogal], lactose [β-D-gal(1-->4)glc] and (2R)-glycerol-O-β-D-galactopyranoside. Purified lectin preparations from both tissues appear to be heterogeneous, containing at least two proteins. For the area vitellina lectin preparation, the molecular weights of these two proteins, estimated under reducing conditions, are approximately 14,200 for a more prominent low molecular weight protein band and 68,100, for the high molecular weight species. In the area vasculosa lectin preparation the molecular weights of these proteins are 13,700 and 68,700. In preparations obtained from the area vitellina, the high molecular weight protein sometimes appears as a doublet with molecular weights of about 71,300 and 62,700. The approximate isoelectric points of a major band determined for each lectin preparation are 6.58, for the area vitellina and 6.65, for the area vasculosa. In addition two to four minor bands in the acidic pH range of 4.1 to 5.5 for the area vitellina lectin and 4.05 to 5.1 for the area vasculosa lectin, could be detected.

The lectins from the yolk sac tissue are similar to a group of soluble, β-D-galactoside-binding lectins isolated from the tissues of a number of vertebrates, including the electric eel (Teichberg et al., 1975; Levi and Teichberg, 1981), calf (Briles et al., 1979), rat (Gartner and Podleski, 1975, 1976; Powell, 1980) and chicken (Den and Malinzak, 1977; Nowak et al., 1977; Kobiler et al., 1978). These soluble, endogenous lectins share a dimeric structure and similar subunit molecular weights in the range 10,000 - 16,500. They also have a requirement for a reducing agent to maintain carbohydrate-binding activity and appear not to require the presence of divalent cations.

In the methods used to extract lectin activity in this study, the requirement for lactose suggests that the binding of an appropriate saccharide to the lectin's active site facilitates extraction. The lectin is possibly bound to some component within the tissue, such as an endogenous ligand, which is displaced by lactose during homogenization. The binding of lactose to the active site of the lectin also appears to be important in maintaining the activity of the lectin. This may be by stabilizing the structure of the lectin or by protecting its active site. The requirement for lactose in the extraction of lectin activity has been reported previously for the chicken embryonic pectoral muscle lectin (Nowak et al., 1977) and the lectin of the eel electric organ (Levi and Teichberg, 1981). The apparent requirement for a reducing agent to maintain the haemagglutination activity of the lectins studied here, may be explained by similar factors to those reported for the eel lectin. Levi and Teichberg (1981), proposed that the reducing agent was necessary to prevent the oxidation of a tryptophan amino acid residue determined to be present in the lactose binding site of the lectin. The reducing agent is believed to function by reducing molecular oxygen present in solution, thus preventing it from oxidizing the tryptophan. These workers determined that exposure of the lectin to oxygen destroys its haemagglutination activity. However since neither the amino acid composition of the lectin nor the oxidizing effect of oxygen were determined in the present study, it is not possible to say if this is the case for the lectins studied here. There appears not to be a requirement for divalent cations for the activity of lectins from the

yolk sac. This was determined on the basis of the efficient extraction and maintenance of the lectins carbohydrate-binding activity in the presence of EDTA, over a period of time in storage. Other galactose-specific vertebrate lectins appear to require the presence of calcium ions to maintain their carbohydrate-binding activity including the lectin of Xenopus laevis embryos (Harris and Zalik, 1982).

The presence of lectin activity was detected by testing if the tissue extracts agglutinated erythrocytes and by showing that the agglutination was saccharide specific i.e. inhibited by simple saccharides. However the accuracy of the assay is somewhat limited by the visual determination of end point of agglutination which to a certain extent is subjective. External conditions of the assay such as temperature and cell number can affect the agglutination reaction (Lis and Sharon, 1981). Other limitations of the assay include, the possibility that agglutination is being measured in extracts of variable purity which may contain more than one specific agglutinin and the limited information on the mechanism of agglutination. The agglutination reaction can be influenced by the concentration of lectin in the extracts and the strength of saccharide binding. In addition the accessibility, distribution and mobility of receptor sites also affect this reaction (Nicolson, 1974).

The ability of the lectin to agglutinate cells suggests that the protein is at least divalent with regard to its carbohydrate-binding sites. Both the valency and the carbohydrate-binding specificity of the lectin are critical for its biological activity. The specificity of the yolk sac lectins has been defined in terms of the saccharide that

inhibits lectin mediated haemagglutination at the lowest concentrations. It has been assumed in the past that the best inhibitor is simlar or even identical to the lectin receptor and that terminal saccharide residues are recognized. However it is now known that a lectin inhibitor in solution may only be mimicking another carbohydrate in the receptor site on the cell surface. The actual interaction between lectin and receptor probably involves complex oligosaccharide structures. And the interaction may be influenced by the nature of the saccharide linkages, the side chains and the protein to which the oligosaccharide chain is attached. In addition the saccharide to which a lectin binds may not be in a terminal position in the glycoprotein receptor (Nicolson, 1974).

Although the interpretation of the saccharide inhibition studies is complicated by the fact that lectin specificity is probably not directed against simple saccharides, some information can be obtained from the results of these studies. The two most potent inhibitors of lectin mediated haemagglutination were the disaccharides, TDG and lactose, which both bear terminal, non-reducing β-D-galactosyl residues. This suggests that the lectin has an affinity for galactoside groups. RGG is also a relatively potent inhibitor of lectin mediated haemagglutination which again suggests the probable importance of galactoside groups. The inhibitory capacity of RGG for β-D-galactoside-binding lectins has not been reported previously. This sugar was selected for use in saccharide inhibition studies because it exhibits both a high specificity and high affinity for the galactose-binding protein present in the β-

methylgalactoside (MeGal) transport system of Escherichia coli (Boos, 1974, 1982). Galactose is not as effective an inhibitor as TDG, lactose or RGG, suggesting that the interaction between the lectin's active site and the sugar, probably involves more than just the terminal galactose group. Possibly the conformation of the oligosaccharide chain or the way in which the reactive sugar is presented to the lectin's active site may play an important role in the interaction. With this in mind, it is interesting to note that α-D-melibiose is a much less effective inhibitor than lactose. The differences in the steric configuration of these two disaccharides may be the reason for the differences in inhibitory capacity. The possible importance of the anomeric configuration of the galactoside is also suggested by the differences in the inhibitory capacities of TDG, lactose and α-D-melibiose. The anomeric specificity of the lectin would appear to be for the β-anomer of galactose. This is suggested by the relatively low concentrations of TDG and lactose needed to inhibit lectin mediated haemagglutination as compared to the relatively higher concentrations of α-D-melibiose. However when methyl-α-D-galactopyranoside and methyl-β-D-galactopyranoside are examined for their inhibitory capacities, lectin extracts from the area vasculosa appear to interact equally well with both anomeric configurations. On the other hand, lectin extracts from the area vitellina appear to interact with the α-anomer at somewhat lower sugar concentrations than with the β-anomer. The latter results have been reported previously for both the chick blastoderm lectins (Zalik et al., 1983) and the chick pectoral muscle lectin (Nowak et al.,

1977). The significance of the results obtained with these alkyl-α and β-glycosides is not known.

The absence of significant inhibition by GalNAc suggests that the hydroxyl group at the carbon-2 position of the saccharide may be important. In addition, the hydroxyl group at the carbon-4 position of galactose appears to be critically involved in lectin binding, since sugars with a galactoside configuration were able to inhibit lectin mediated haemagglutination. The absence of significant inhibition by L- and D-fucose further suggests the importance of the hydroxyl groups at the carbon-2, carbon-4 and carbon-6 positions of galactose.

The differences in the concentrations of sugars necessary to inhibit lectin mediated haemagglutination by lectins from the area vasculosa and area vitellina may be due to several factors including: a) subtle differences in the active sites of the lectins from the two sources; b) the way in which the lectin subunits aggregate, which may subtly affect sugar binding; c) the non-specific interaction of contaminating material present in the preparation with the lectin molecules, which may interfere with sugar binding; and d) the limitations of the assay, since different saccharide preparations were used in repeat experiments. It is also possible that the different lectin preparations may have been of variable purity and may have contained more than one specific agglutinin. This factor appears to be of importance since it was determined that the purified lectin fractions were not homogeneous on SDS-polyacrylamide gel electrophoresis performed under reducing conditions. Both lectin preparations contained at least

two distinct proteins. The relative contribution of these two proteins to the binding of the different saccharides could not be determined. This is assuming that both proteins have carbohydrate-binding abilities. Further, the specific activity of the lectin from the area vitellina tissue was one-quarter that of the area vasculosa lectin preparations. This may indicate that up to 75 percent of this preparation is contaminant or that the lectin of the area vitellina is more susceptible to inactivation.

The conclusions drawn from these saccharide inhibition studies are that the lectin preparations from the area vitellina and the area vasculosa are essentially the same in their affinity for saccharides bearing a β-D-galactoside configuration. Their pronounced affinities for TDG and lactose are comparable to the results obtained for the chick blastoderm lectins (Zalik et al., 1983). Lectin activity from 16-day embryonic chick pectoral muscle tissue also showed similar β-D-galactoside-binding affinities which has been reported previously (Nowak et al., 1977).

As mentioned previously, the purified lectin preparations from the area vitellina and the area vasculosa of the 2-day yolk sac were determined to be heterogeneous on reducing SDS-polyacrylamide gel electrophoresis since at least two proteins were present in the preparations. Because of the low stainability with Coomassie blue which was used to stain gels initially, protein bands were subsequently visualized with a silver stain. Under the conditions of the silver staining method, relatively strong staining was achieved. In the Coomassie blue staining procedure, the low molecular weight protein

appeared to be somewhat more prominent than the high molecular weight species. This may indicate the presence of higher amounts of this protein in the lectin preparations. Although staining of the proteins with silver is more sensitive than with Coomassie blue, staining intensity does not appear to be stoichiometric (Poehling and Neuhoff, 1981). And it was not possible to determine differences in the relative amounts of protein present in the two bands based on the intensity of silver staining.

When purified lectin samples were examined under non-reducing conditions, the high molecular weight protein observed under reducing conditions was not present. Instead a large protein with an approximate molecular weight of 127,000 for the area vitellina preparation and 124,000 for the area vasculosa preparation, sometimes appeared. This protein may represent a stabilized aggregate of the high molecular weight protein which can be visualized under reducing conditions. In the presence of SDS, urea and 2ME i.e. under reducing conditions, this aggregate may dissociate to give the high molecular weight protein (MW 68,100 for a. vit. and MW 68,700 for a. vasc.). The low molecular weight protein species was always observed under non-reducing conditions.

It was not possible to determine from these experiments if only one of the two proteins (present under reducing conditions) contains lectin activity or if both proteins are lectins with similar galactoside-binding properties. One possibility is that the high molecular weight protein detectable under reducing conditions, is a stabilized aggregate

of the low molecular weight protein. Or this high molecular weight protein could be a precursor molecule that gives rise, by cleavage, to the low molecular weight protein and the latter may be the active lectin. It is also possible that the lectin activity is present as an aggregate formed by these two components visualized under reducing conditions. Lis and Sharon (1981) in a review on plant lectins, have suggested that lectins may be composed of subunits which can undergo complex association and dissociation reactions that result in multiple forms of the same lectin which differ in molecular size.

Under non-dissociating conditions and in the presence of lactose, aqueous solutions of the crude lectin preparations from the area vitellina and the area vasculosa appeared to be present in several oligomeric forms. This is in contrast to other chicken β-D-galactoside-binding lectins which apparently exist as dimers (Nowak et al., 1977; Kobiler et al., 1978). However, purified lectin preparations were used in these latter gel filtration studies. The yolk sac lectins reported in their study are more similar to the chick blastoderm lectins in their heterogeneous nature on gel filtration chromatography (Zalik et al., 1983). The molecular weight range in which crude lectin from area vitellina extracts was present suggests that the low molecular weight component of the preparation may be present in several oligomeric forms. Whereas in the crude lectin preparation from the area vasculosa, this component may be present in both oligomeric and monomeric forms. The high molecular weight component of both preparations may be present either as a monomer or in aggregate forms which have lost their haemagglutination activity. These possibilities are suggested by the

absence of activity in the molecular weight ranges which would indicate that this protein is multimeric. The recovery of lectin in these studies was very low for both crude lectin preparations which could be due to inactivation of lectin activity. Because of the low recovery of activity in these experiments no definite conclusions can be drawn at the present time.

The yolk sac lectins reported in this study have properties in common with the chick blastoderm lectins reported by Zalik et al. (1983). These properties include the ability to agglutinate trypsinized rabbit erythrocytes, similar saccharide-binding affinities, similar subunit molecular weights and a similar heterogeneous nature on gel filtration chromatography. The blastoderm lectins, $LI_{(s)}$ (soluble form) and $LII_{(p)}$ (particle-associated form) have estimated molecular weights of 11,000 and 70,000 and 72,000 (a doublet) respectively. These two lectins have been isolated from the area opaca tissue of primitive streak stage embryos. Both lectins are present in the epiblast (ectoderm) and endoderm of this tissue but the $LII_{(p)}$ is present in the endoderm at much higher levels than the $LI_{(s)}$. These two lectins also appear to be distinguishable on the basis of the method of extraction from the area opaca tissue. A possible function for the blastoderm lectins in the cell rearrangements and early epibolic processes during gastrulation has been suggested (Milos and Zalik, 1982; Zalik et al., 1983).

In this study lectin activity is detectable in the area vasculosa and area vitellina of the developing yolk sac. Generally higher levels

of activity are present in the area vasculosa tissue extracts. When extracts of these two areas were analyzed, the differences in lectin activity were significant. During development of the yolk sac, the extension of the area vitellina (composed of ectoderm and endoderm) over the yolk surface is very rapid between three and five days of development. At the same time the area vasculosa is growing by invasion of the area vitellina by the mesodermal sheet of cells. The presence of lectin activity and changes in the levels of this activity in the two areas appears to coincide with these events in the growth and differentiation of the yolk sac. During the earliest stages of development, the cells of the endodermal layer are undergoing extensive rearrangements involving the breaking of contacts and formation of new contacts between cells. These changes occur as the single layer of differentiated, endodermal epithelium is formed. An irregularly stratified zone of cells is recognizable as the inner boundary of the area opaca at the primitive streak stage of embryo development (Romanoff, 1960). By the end of the second day of chick incubation, this zone of cells has widened considerably and is moving distal to the area vasculosa, to form part of the inner region of the area vitellina. With development this zone of stratified cells continues to spread centrifugally by the addition of cells from the periphery, while cells at its medial border differentiate to form an epithelium.

Lectin activity is present in the area vitellina where the endodermal cells in a stratified layer are undergoing rearrangements as the transition to area vasculosa takes place. Also, a large proportion of this area consists of cells that are just being formed and which are

dispersed among yolk granules. Lectin activity is also present, at higher levels, in the region of the area vasculosa where cells are becoming more differentiated as they form the epithelial lining of the yolk sac. The results of this study appear to indicate that at times when the endodermal cells of the yolk sac are undergoing differentiation, relatively high levels of lectin activity are present. At later developmental stages when the endodermal lining of the yolk sac is almost completely formed, levels of lectin activity in the area vasculosa are similar to those in the area vitellina.

In the tissue examined in the present investigation although significant changes in lectin activity were found at some developmental stages, these changes are not as dramatic as those reported for the embryonic chick pectoral muscle lectin between 8 and 16 days of development (Nowak et al., 1976; 1977). It would be important to examine the lectin activity of the yolk sac tissue at later developmental stages to determine if changes in lectin activity occur in this tissue with further development. This is because by the twelfth day of chick incubation, the stratified layer of endodermal cells has been completely transformed into epithelium and is fully differentiated and the area vasculosa continues to grow, up until the fifteenth day (Romanoff, 1960).

This study has shown that lectin activity is present in the developing yolk sac of chicken embryos. It is possible that the β-D-galactoside-binding lectin activities present in the two-day yolk sac are similar to those found in the primitive streak stage area opaca.

And the lectin may play a role in the extensive cell rearrangements that occur during the epibolic processes of yolk sac formation. Future considerations could further elucidate this point. Such considerations would include the isolation of the lectin activity from the endodermal layer of cells, particularly in the region where the cells are being transformed from an irregular, multilayered arrangement to an epithelium of closely packed, columnar cells. In addition, the immunohistochemical localization of the lectin activity would be necessary to determine if this lectin is present on the surface of the cells involved in the formation of the yolk sac epithelium. Other considerations include determining the carbohydrate-binding activity of the two protein components, observed in reducing SDS-gels, from purified lectin preparations of the area vitellina and area vasculosa and elucidating their relationships to each other.

There are other possible functions for the carbohydrate-binding activity detected in extracts of the early yolk sac membrane of the chick embryo which need to be investigated. Some involvement in the absorption of nutrient proteins from the yolk is possible. A major function of the yolk sac membrane is the transport of protein precursors from the yolk to the growing embryo. And there is indirect evidence suggesting that the yolk sac membrane has the ability to transport proteins intact from the yolk and then possibly to the embryo. One study has suggested that the area opaca or presumptive yolk sac membrane of chick embryos, in culture, is the specific site of ovalbumin uptake (Hassell and Klein, 1971). In addition, this study indicated that ovalbumin was primarily degraded in the area opaca and was not

transported intact to the embryo. Instead the breakdown products of ovalbumin are thought to be transported to the embryo via the circulatory system or they may be used in the synthesis of embryonic serum proteins by the yolk sac, which are then transported to the embryo. Ovalbumin possesses galactose-containing sugar chains (Yamashita et al., 1978) and there is the possibility that β-D-galactoside-binding lectins in the endodermal cells of the yolk sac might be involved in the binding and/or uptake of this protein from the yolk.

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